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13. Abstract (Maximum 200 Words) *(abstract should contain no proprietary or confidential information)*
An overriding conclusion of the work of the past 2 years on this project is that ethanol at concentrations observed in problem drinkers and alcoholics (e.g., an individual consuming either 6 beers, or 6 one shots of whiskey, or 6 glasses of wine) has a multifaceted effect on cellular regulation of cholesterol. We show that several of the systems involved in regulating cholesterol transport are perturbed by ethanol (HDL, apoA-I, LDL, PC-PLC, PC-PLD). The Golgi complex plays an important role in protein and lipid trafficking and we show that it is a target of ethanol. We are now able to measure the distribution of cholesterol in different Golgi regions and this method will be used to elucidate effects of ethanol on Golgi complex function. 25 mM ethanol was just as effective in stimulation of PC-PLD activity and LDL uptake as were higher ethanol concentrations. However, 25 mM ethanol had less of an effect on HDL or apoA-I mediated cholesterol efflux than higher ethanol concentrations. These results raise the question as to whether ethanol concentrations lower than 25 mM would have a similar or greater effect on PC-PLD activity and LDL uptake and would such effects be protective of cell function.

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ABBREVIATIONS

ApoA-I, apolipoprotein A-I

CHD, coronary heart disease

DHE, dehydroergosterol

G1, gradient fraction 1

G2, gradient fraction 2

G3, gradient fraction 3

HASMC, human aortic smooth muscle cells

HBBS, HEPES buffer saline solution

HCAEC, human coronary artery endothelial cells

HDL, high density lipoprotein

LDL, low density lipoprotein

PA, phosphatidic acid

PC-PLC, phosphatidylcholine-phospholipase C

PC-PLD, phosphatidylcholine-phospholipase D

Petoh, phosphatidylethanol

TNS, trypsin neutralizing solution

INTRODUCTION

Risk of coronary heart disease (CHD) morbidity and mortality is reduced by moderate alcohol consumption whereas some studies suggest that heavy alcohol consumption has little or no benefit for CHD[1-4]. A generally accepted finding of epidemiological and experimental studies is that high-density lipoproteins (HDL) levels are increased in association with alcohol consumption [3;5-7]. An increase in HDL has been proposed to be one of the potential factors involved in the protective effects of moderate alcohol consumption on CHD [5;8]. HDL levels however, are increased in heavy drinkers but their risk of CHD is higher than that of moderate alcohol drinkers [9-11]. An important function of HDL is removal of excess lipids including cholesterol from cells and evidence has shown that chronic and acute administration of ethanol alters lipoprotein-mediated cholesterol efflux. Cholesterol efflux was decreased in mouse macrophages incubated with HDL of human alcoholic subjects [12]. However, it was recently reported that cholesterol efflux was stimulated from Fu5AH rat hepatoma cells to plasma of human subjects who consumed 40 g of alcohol per day for 3 weeks [13]. Acute administration of a very high ethanol concentration (350 mM) increased the rate of cholesterol efflux from erythrocytes to plasma [14]. Ethanol concentrations (25 and 50 mM) that are seen in alcoholics and heavy drinkers inhibited cholesterol efflux from rat fibroblasts to HDL and apoA-I [15]. Molecular mechanisms that are involved in effects of moderate and heavy alcohol consumption on cholesterol transport and cellular cholesterol distribution are not well-understood. We propose that ethanol modifies high density lipoprotein (HDL)-mediated cholesterol efflux from cells and low density lipoprotein (LDL)-mediated cholesterol influx into cells. These actions of ethanol alter the intracellular distribution of cholesterol leading to cellular dysfunction. Potential mechanisms that may explain effects of ethanol on cholesterol transport and distribution include ethanol perturbation of lipoprotein structure, LDL receptor function and expression, perturbation of the Golgi complex and signaling pathways such as phosphatidylcholine-phospholipase C (PC-PLC) and phosphatidylcholine-phospholipase D (PC-PLD) that are involved in reverse cholesterol transport. These potential mechanisms are being studied in this grant that consists of three major objectives: **Objective 1.** to determine mechanisms of ethanol-induced increase in LDL receptor-mediated cholesterol influx; **Objective 2.** to determine mechanisms of ethanol-induced alterations in the intracellular distribution of cholesterol; and **Objective 3.** to determine mechanisms of ethanol-induced perturbation of HDL-mediated cholesterol efflux from cells. Studies are being conducted using a combination of fluorescence spectroscopy, confocal microscopy and cell culture techniques. Human aortic smooth muscle cells and human endothelial cells are being used in the experiments.

BODY OF REPORT

Ethanol Inhibits Apolipoprotein A-I Mediated Cholesterol Efflux from Human Aortic Smooth Muscle Cells (HASMC) and Human Coronary Artery Endothelial Cells (HCAEC).

The purpose of these experiments was to determine if acute ethanol administration altered cholesterol efflux induced by apoA-I in cells that are directly involved in cardiovascular function. HASMC and HCAEC were used in this study. Confluent cells were preloaded with the fluorescent cholesterol analogue, dehydroergosterol (DHE) (3 μg/ml of cell culture medium) for 18 h in 0.5% lipoprotein deficient serum. DHE incubation time and concentration were determined from sampling incorporation of DHE into cells over 0-24 h. Cells were incubated for

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a period of 30 min with 0, 25, 50, or 75 mM ethanol after which 30 µg of apoA-I/ml of medium was added and cells were further incubated for 2 h. Cells were then washed twice with HBBS, trypsinized and neutralized with TNS or serum, collected, and centrifuged at 14,000 rpm for 1 min in an Eppendorf centrifuge (model 541 7R). The pellet containing cells was washed twice with 1 ml PBS and homogenized in a loose-fitting homogenizer. The protein of the homogenized samples was determined by Bradford method. Sample (25µg protein) was placed in a quartz cuvette and DHE fluorescence intensity determined using a LS-50B fluorimeter (Perkin-Elmer, Norwalk, CT) with the excitation wavelength of 324 nm and emission wavelength of 376 nm.

There was a 24% reduction in sterol in HASMC when incubated with apoA-I alone as compared with cells not incubated with apoA-I and this difference was significant ($p \leq 0.0002$) (Figure 1). Ethanol significantly ($p \leq 0.001$) inhibited apoA-I induced sterol efflux beginning at a concentration of 25 mM ethanol (Figure 1). In the presence of 50 and 75 mM ethanol, there was only approximately 6% and 3% of sterol removed respectively, by apoA-I.

There was a 36% reduction of sterol in HCAEC by apoA-I (Figure 2) as compared to control cells in the absence of apoA-I and this difference was significant ($p \leq 0.005$). Cholesterol efflux was significantly ($p \leq 0.005$) inhibited in the presence of 25 mM ethanol in contrast to apoA-I alone (Figure 2). Cholesterol efflux to apoA-I was abolished at ethanol concentrations of 50 and 75 mM (Figure 2).

Ethanol Stimulates PC-PLC and PC-PLD in HASMC. Both PC-PLC and PC-PLD appear to be two of the mechanisms involved in regulating cholesterol efflux from cells [16]. PC-PLD is of particular importance with respect to action of ethanol because in the presence of alcohols there is a transphosphatidylation reaction and a phosphatidylalcohol is formed and a reduction of PA production [17;18]. PA has been shown to stimulate cholesterol efflux [16]. Ethanol may alter activity of PC-PLC and PC-PLD that could impact on cholesterol efflux. Effects of ethanol on activation of PC-PLC and PC-PLD activity were determined in HASMC using an enzyme coupled assay and N-acetyl-3,7-dihydroxyyphenoxazine using the Amplex red PC-PLC and PC-PLD assay kits (Molecular Probes, Eugene, OR)[19]. Cells were incubated with ethanol (0, 25, 50, and 75 mM) for 2 h after which time cells were then washed twice with HBBS, trypsinized and neutralized with TNS or serum, collected, and centrifuged at 14,000 rpm for 1 min in an Eppendorf centrifuge (model 541 7R). The pellet containing cells was washed twice with 1 ml PBS and homogenized in a loose-fitting homogenizer and aliquots placed in 96 well-plates and the assay reagents added. The intensity of the Amplex Red complex formed was measured after 60 min in a fluorescence microplate reader (Spectra Max Gemini XS, Molecular Device, Sunnyvale, CA) using an excitation wavelength of 542 nm and an emission wavelength of 590 nm. PC-PLD was significantly stimulated by ethanol (Figure 3). There was a 2-fold increase in PC-PLD activity by 25 mM ethanol as compared to control cells ($p \leq 0.0001$). It can be seen in Figure 3 that both 50 and 75 mM ethanol significantly ($p \leq 0.004$) increased PC-PLD activity as compared to control cells but this effect was certainly not as large as observed for 25 mM ethanol. Activity of PC-PLC was significantly ($p \leq 0.02$) increased by 25 mM ethanol as compared to the control cells resulting in a 16% increase in activity of the enzyme (Figure 4). PC-PLC activity was higher in the presence of 50 or 75 mM ethanol as compared to control but these differences were not significant. The increase in activities of PC-PLD and PC-PLC by ethanol is in agreement with earlier reports in studies of hepatocytes and fibroblasts [20;21]. An ethanol concentration of 25 mM

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had a greater effect on activity of both phospholipases as compared with 50 and 75 mM ethanol particularly with respect to PC-PLD.

Effects of Propranolol on ApoA-I induced Cholesterol Efflux from HASMC. Hydrolysis of PC by PC-PLD produces phosphatidic acid (PA) and choline. In the presence of an alcohol, PC-PLD catalyzes a transphosphatidylation reaction where PC is transferred to an alcohol that yields phosphatidylalcohol. When ethanol is present, phosphatidylethanol (Petoh) is formed. Propranolol (100 µM) blocks PA phosphohydrolase resulting in an increase in PA and stimulates cholesterol efflux [16]. We determined if propranolol (100 µM) would stimulate cholesterol efflux to apoA-I and if ethanol (50 mM) would interfere with this process in HASMC. Figure 5 shows that apoA-I significantly ($p \leq 0.01$) removed cholesterol from cells as compared to the control condition where apoA-I was not present and this finding is consistent with data shown in Figure 1 in HASMC. Addition of propranolol enhanced apoA-I mediated cholesterol efflux by 20% but this difference did not reach statistical significance when compared with apoA-I alone (Figure 5). Ethanol (50 mM) inhibited the actions of propranolol and it can be seen that the level of cholesterol in those cells was similar to the control condition where apoA-I was not present. These experiments need to be repeated using a larger sample size and it needs to be determined as to the levels of phosphatidylethanol (Petoh) in comparison to PA produced at the ethanol concentrations used in the present studies. Petoh could interfere with vesicular trafficking from the Golgi complex to the plasma membrane. Another possibility is if the amount of PA is reduced by the ethanol concentrations used in the present experiments then PA may directly contribute to cholesterol efflux. It has been proposed that PA may function as a second messenger in cholesterol transport [16].

Cholesterol Distribution in the Golgi Complex. We had previously shown that ethanol reduced cholesterol content in the Golgi complex of fibroblasts as revealed by confocal microscopy and the fluorescent cholesterol analogue, NBD-cholesterol and a fluorescent marker for the Golgi complex, BODIPY TR ceramide [22]. The Golgi complex consists of different regions and it has been reported that there is a gradient of cholesterol from the *cis* region to the *trans* region of the Golgi complex. We have applied a method in our lab to isolate different Golgi regions using sucrose density centrifugation. Our initial studies were with an immortalized astrocyte cell line because these cells are provided to our lab at little or no costs. Subsequent studies will be performed using the more expensive HASMC and HCAEC cells. Cells were harvested and washed twice with PBS. The cells were suspended in G-buffer (10 mM Tris-HCl, 0.25 M Sucrose and 2 mM $MgCl_2$, pH 7.4) containing 10 mM $CaCl_2$. Leupeptin and PMSF were added to the cell suspension to inhibit proteolytic enzymes, prior to homogenization. The cells were homogenized in a Potter-type homogenizer and homogenized with 20-30 strokes using a serrated homogenizing pestle. The homogenate was centrifuged at 5,000 x g for 10 min. The pellet was suspended and homogenized in 1.4 M sucrose and over layered with 0.8 M, 1.0 M, 1.2 M sucrose gradients in ultracentrifuge tubes. Samples were then centrifuged at 95,000 x g for 2.5 h in a SW28 rotor in a Beckman L8-70 ultracentrifuge. Each interface was carefully removed and diluted with G buffer and centrifuged in a SS34 rotor at 45,900 x g for 20 min. Each pellet was suspended in buffer and used for the experiments. Protein concentrations of the fractions were determined using Bradford assay. Cholesterol content in each fraction was determined enzymatically in a microassay using the Boehringer-Mannheim diagnostic kit [23] and procedures reported by our laboratory [24-26].

Figure 6 shows that the two fractions thought to represent the *trans* Golgi (G1) and the *cis*-medial region (G2) differ in cholesterol content. The G1 fraction had significantly ($p \leq 0.0001$) more cholesterol as compared with any of the other fractions. A similar distribution of cholesterol was found using the fluorescent cholesterol analogue, NBD-cholesterol (data not shown). Cholesterol content was similar in the G2 and G3 fractions although we did find that enrichment of the Golgi complex marker galactosyltransferase was greater in the G1>G2>G3 (data not shown). Regions of the Golgi complex may be differentially acted upon by ethanol and this possibility will be examined in the next grant year.

Ethanol Stimulates Uptake of LDL into HCAEC and HASMC. Cellular cholesterol is regulated by activity of HMG-CoA reductase, reverse cholesterol transport and uptake of cholesterol into cells. We have found that ethanol at concentrations observed in alcoholics and heavy drinkers inhibited cholesterol efflux to HDL and apoA-I in HASMC. Not only is cholesterol efflux affected by ethanol but uptake of cholesterol into cells was altered by ethanol. Data were presented in the previous report showing that ethanol stimulated uptake of LDL into rat fibroblasts and that work has been extended to human vascular cells. In preliminary experiments, effects of ethanol on the uptake of fluorescent labeled LDL were examined in HASMC and HCAEC using confocal microscopy and fluorescence spectroscopy, Cells were incubated with different concentrations of ethanol (0, 25, 50, and 75 mM) for 1, 2.5 and 5 h in the presence of fluorescent-labeled LDL in media that was lipoprotein-deficient. Figure 7 shows confocal images of HCAEC and HASMC incubated with different concentrations of ethanol for 5 h. The increase in the red intensity (BODIPY) and green intensity (DIL) intensity denotes an increase in the uptake of fluorescent-labeled LDL. Ethanol clearly stimulated uptake of LDL into HCAEC and HASMC (Figure 7).

Fluorescence intensity was quantified by measuring intensity in separate samples using a LS 50-B fluorometer. Effects of ethanol on LDL uptake were most pronounced after 5 h of incubation in both cell types (Figures 8 & 9). Interestingly, effects of ethanol on LDL uptake were not concentration dependent. LDL uptake was similar at each ethanol concentration in HCAEC and HASMC (Figures 8 & 9). Uptake of LDL into cells may be particularly sensitive to effects of ethanol and significant uptake might be observed at ethanol concentrations lower than 25 mm. LDL uptake in HCAEC increased over time in the control cells (Figure 8). In HASMC the only increase in the control condition was at 2.5 h (Figure 9). It is important to point that the data are based on an n of 1 for confocal microscopy and an n of 1 for the fluorimeter experiments and will have to be repeated. However, the fact that LDL uptake was stimulated by ethanol in the 5 h incubation in both cell types and in separate experiments adds to the reliability of the results. Potential explanations for the stimulatory effects of ethanol on LDL uptake may reside in ethanol-induced inhibition of HMG-CoA reductase that could lead to upregulation of the LDL-receptor. Statins inhibit HMG-CoA reductase resulting in an increase in the number of LDL-receptors. Ethanol may directly induce upregulation of the LDL-receptor. These potential effects of ethanol will be examined in the next grant year.

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KEY RESEARCH ACCOMPLISHMENTS

- Ethanol significantly inhibited cholesterol efflux from human aortic smooth muscle cells and human coronary artery endothelial cells to apoA-I. ApoA-I mediated cholesterol efflux was more disrupted by ethanol than was HDL-mediated cholesterol efflux.
- Activity of both PC-PLC and PC-PLD in human aortic smooth muscle cells was stimulated by ethanol with PC-PLD activity being most affected.
- Cholesterol distribution in different regions of the Golgi complex as revealed by two different methods and provides a precise determination of ethanol perturbation of Golgi complex function.
- Ethanol stimulated uptake of LDL into human aortic smooth muscle cells and human coronary artery endothelial cells.
- A low concentration of ethanol was more effective in stimulating activity of PC-PLD and PC-PLC in cells as compared with higher ethanol concentrations. This concentration dependent effect was also observed with LDL uptake but not either HDL or apoA-I mediated cholesterol efflux.

REPORTABLE OUTCOMES

Igbavboa, U., Pidcock, J.M., Malo, T.M., Johnson, L.N.A., Wood, W.G. HDL and apolipoprotein A-I induced cholesterol efflux is inhibited by ethanol in human smooth muscle aortic cells: Roles of phospholipases C and D. *Alcoholism: Clinical and Experimental Research,* 2002, *26,* 60A.

Igbavboa, U., Pidcock, J.M., Malo, T.M., Johnson, L.N.A., Wood, W.G. Cholesterol efflux is inhibited by ethanol in human aortic smooth muscle cells: Differences in sensitivity of HDL and apolipoprotein A-I and the roles of phosphatidylcholine-phospholipase C and D. *Alcoholism: Clinical and Experimental Research,* 2002, submitted.

Igbavboa, U., Pidcock, J.M., Jognson, L.N.A., Malo, T.M., Sun, G.Y., and Wood, W.G. Cholesterol distribution in the Golgi complex of astrocytes is altered by amyloid beta-peptide$_{1-42}$: Roles of peptide structure and phosphatidylcholine-phospholipase D.
Journal of Biological Chemistry, 2002, revised manuscript in preparation.

CONCLUSIONS

Cholesterol is important in regulation of cell structure and function. Equally important is the role that cholesterol plays in vascular disease. The focus of this grant is on mechanisms of alcohol induced disruption of cellular cholesterol transport and distribution. An overriding conclusion of the work of the past 2 years is that ethanol at concentrations observed in problem drinkers and alcoholics (e.g., an individual consuming either 6 beers, or 6 one shots of whiskey, or 6 glasses of wine) has a profound and multifaceted effect on cellular regulation of cholesterol. Our work to date shows that many of the systems involved in regulating cholesterol transport are perturbed by ethanol (HDL, apoA-I, LDL, PC-PLC, PC-PLD). The Golgi complex plays an important role in protein and lipid trafficking and is a target of ethanol and this conclusion is based on our studies with confocal microscopy and PC-PLD activity. We are now able to measure the distribution of

cholesterol in the Golgi complex and this method will be used to elucidate effects of ethanol on Golgi complex function. An interesting set of observations was that 25 mM ethanol was just as effective in stimulation of PC-PLD activity and increasing LDL uptake as were higher ethanol concentrations. On the other hand, 25 mM ethanol had less of an effect on HDL or apoA-I mediated cholesterol efflux as compared with the higher ethanol concentrations employed. The implications of these findings on the effects of 25 mM ethanol with respect to cell function are not clear. However, the results raise the question as to whether ethanol concentrations lower than 25 mM would have a similar or greater effect on PC-PLD activity and LDL uptake and would such effects be protective of cell function.

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Appendices

Figures 1-9

Reprints

Figure Legends

Figure 1. Ethanol Inhibits ApoA-I Mediated Cholesterol Efflux from HASMC. Confluent HASMC were incubated with 3μg of DHE/mL of cell culture medium for 18 h. Cells were then washed twice using DHE-free medium and incubated for 30 min with 25, 50, 0r 75 mM ethanol or no ethanol, after which 30 μg of human plasma apoA-I /mL of medium was added and cells were incubated for 2 h. Cells were then harvested and processed, and DHE fluorescence was measured. The control cells contained DHE but were not incubated with apoA-I or ethanol. Each bar represents DHE content remaining in cells. Data are means ± SEM (n = 3). *p ≤ 0.001 as compared to apoA-I no ethanol; **p ≤ 0.0002 as compared to control no apoA-I.

Figure 2. Ethanol Inhibits ApoA-I Mediated Cholesterol Efflux from HCAEC. Treatment conditions were the same as described in Figure 1. Data are means ± SEM (n = 3). *p ≤ 0.005 as compared to control no apoA-I and apoA-I no ethanol. **p ≤ 0.002 as compared to apoA-I no ethanol.

Figure 3. Ethanol Stimulates PC-PLD Activity in HASMC. Confluent cells were incubated with different concentrations of ethanol for 2 h after which time cells were harvested. 20 μg of cells were placed in 96 well plates and PC-PLD determined using the Amplex$^®$ red PC-specific PLD assay kit. Samples were incubated for 60 min at 37°C. Fluorescence was measured with a microplate reader using an excitation wavelength of 542 nm and emission wavelength of 590 nm. Data are means ± SEM (n = 4). *p ≤ 0.004 as compared to 0 ethanol; **p ≤ 0.0001 as compared to 0, 50 and 75 mM ethanol.

Figure 4. Effects of Ethanol on PC-PLC Activity in HASMC. Confluent cells were incubated with different concentrations of ethanol for 2 h after which cells were harvested. 20 μg of cells were placed in 96 well plates and PC-PLC determined using the Amplex$^®$ red PC-specific PLC assay kit. Samples were incubated for 60 min at 37°C. Fluorescence was measured with a microplate reader using an excitation wavelength of 542 nm and emission wavelength of 590 nm. Data are means ± SEM (n = 4). *p ≤ 0.02 as compared to each condition.

Figure 5. Effects of Propranolol on ApoA-I Mediated Cholesterol Efflux from HASMC. Treatment conditions were the same as described in Figure 1 except cells were incubated with or without 100 μM propranolol for 2 h. Data are means ± SEM (n = 3). *p ≤ 0.01 as compared to control no apoA-I.

Figure 6. Distribution of Cholesterol in the Golgi Complex of Astrocytes. The Golgi complex was isolated using sucrose density centrifugation. The G1 fraction is the band at the 0.8-1.0 M sucrose interface and G2 fraction is the band at the 1.0-1.2 M sucrose interface. The G3 fraction is the band at the 1.2-1.4 M sucrose interface. Cholesterol was determined enzymatically. Values are means ± SEM (n = 3). *p ≤ 0.01 as compared with the homogenate and pellet; **p ≤ 0.0001 as compared with all other fractions.

Figure 7. Confocal Images of HCAEC and HASMC Incubated with Different Concentrations of Ethanol and Fluorescent Labeled LDL. Cells were plated on cell culture chamber slides and grown to 70-80 confluency in EB media at which time media was replaced with lipoprotein-

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deficient serum for 12 h. Cells were then incubated with 300 µl of BODIPY-LDL (0.1 µg/ml) and different ethanol concentrations for 5 h at 37°C at 5% CO_2 atmosphere in an incubator. After 5 h, the slides were washed and fixed with 4% paraformaldehyde and mounted for confocal microscopy. Confocal images were taken using an Olympus BX50 confocal microscope at an excitation wavelength of 568 nm.

Figure 8. Ethanol Stimulates Uptake of LDL into HCAEC. Cells were grown in 25 cm^2 flasks until 70-80% confluent at which time media was replaced with lipoprotein-deficient serum for 12 h. Cells were then incubated with 1ml of BODIPY-LDL (0.1 µg/ml) and different ethanol concentrations. Cells were incubated for 1, 2.5 and 5 h at 37°C and 5% CO_2 atmosphere in an incubator. Cells were washed and harvested and protein measured. Intensity of BODIPY was measured using a LS-50-B fluorimeter at an excitation wavelength of 504 nm and emission wavelength of 522 nm.

Figure 9. Ethanol Stimulates Uptake of LDL into HASMC. Cells were grown in 25 cm^2 flasks until 70-80% confluent at which time media was replaced with lipoprotein-deficient serum for 12 h. Cells were then incubated with 1ml of DIL-LDL (0.1 µg/ml) and different ethanol concentrations. Cells were incubated for 1, 2.5 and 5 h at 37°C in a 5% CO_2 atmosphere in an incubator. Cells were washed and harvested and protein measured. Intensity of DIL was measured using a LS-50-B fluorimeter at an excitation wavelength of 555 nm and emission wavelength of 571 nm.
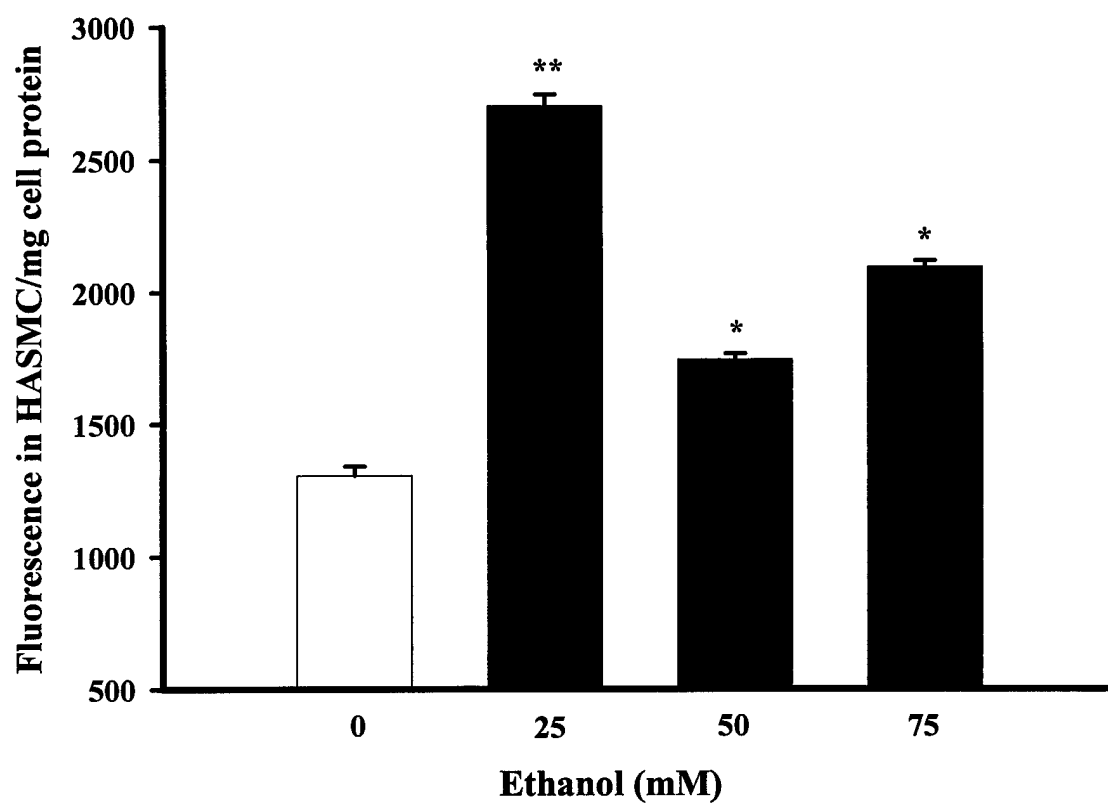
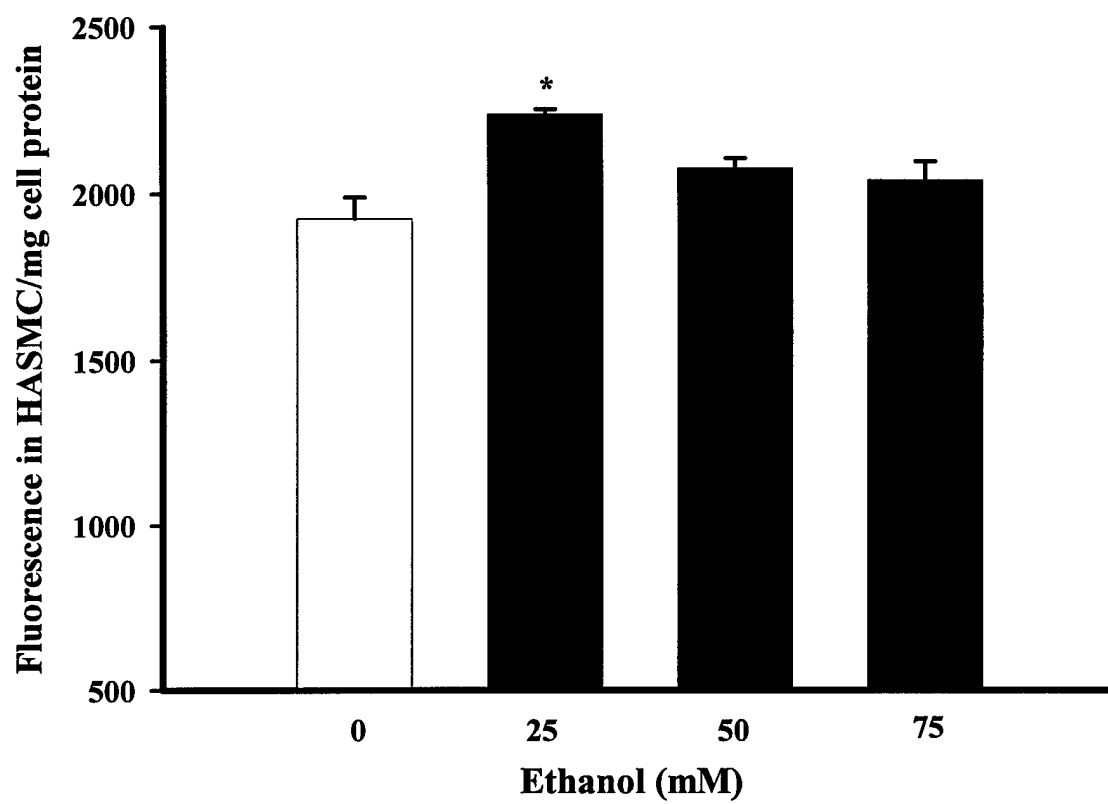Figure 1. Ethanol Inhibits ApoA-I Mediated Cholesterol Efflux from HASMC
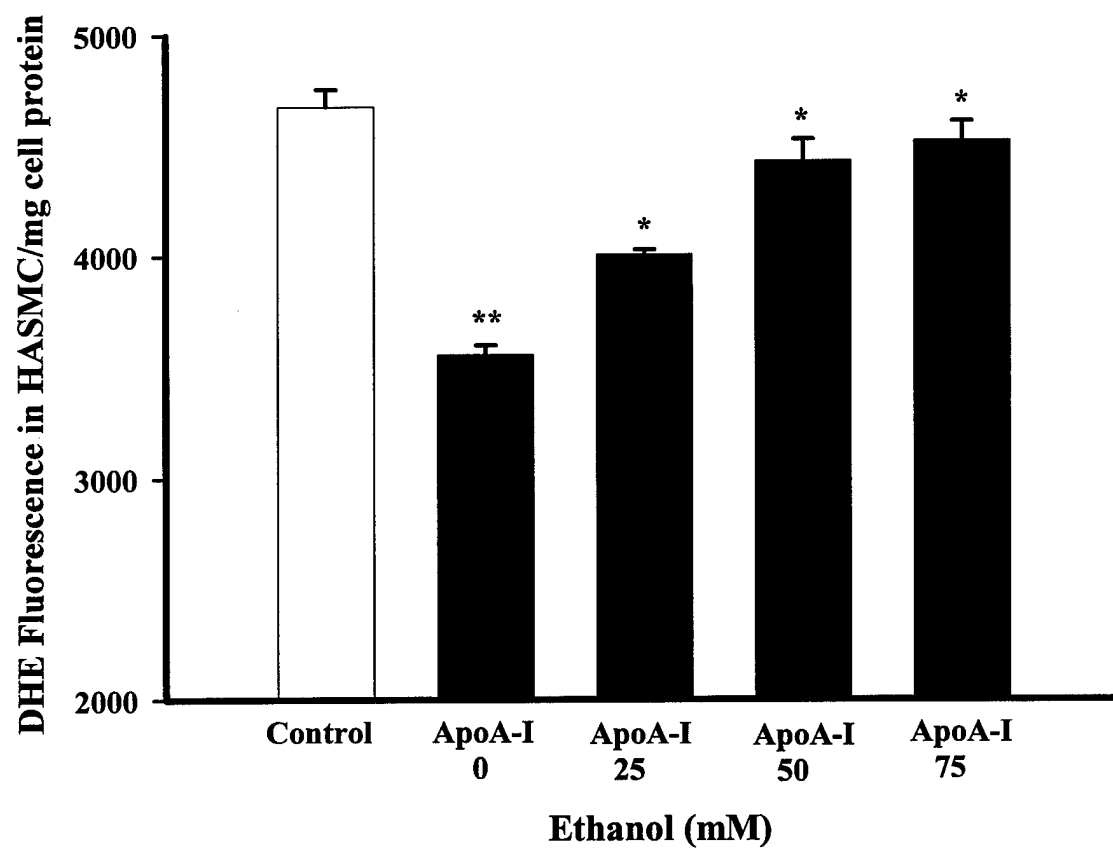
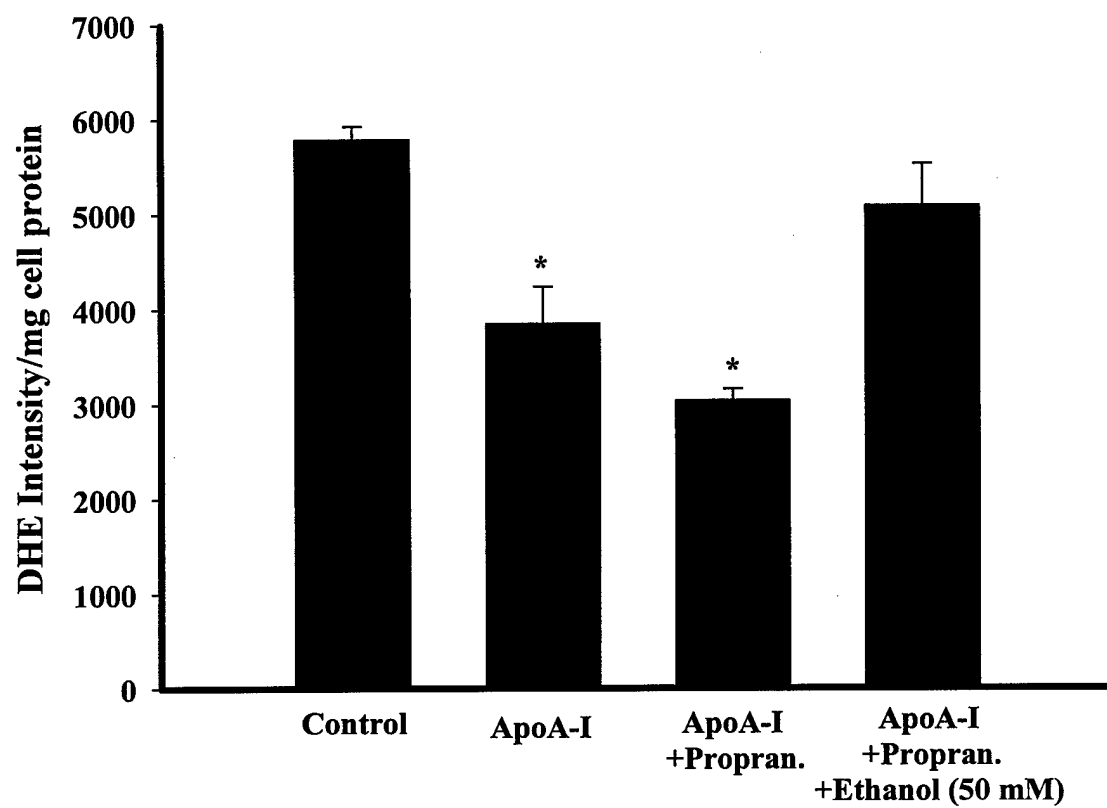**Figure 2. Ethanol Inhibits ApoA-I Mediated Cholesterol Efflux from HCAEC**
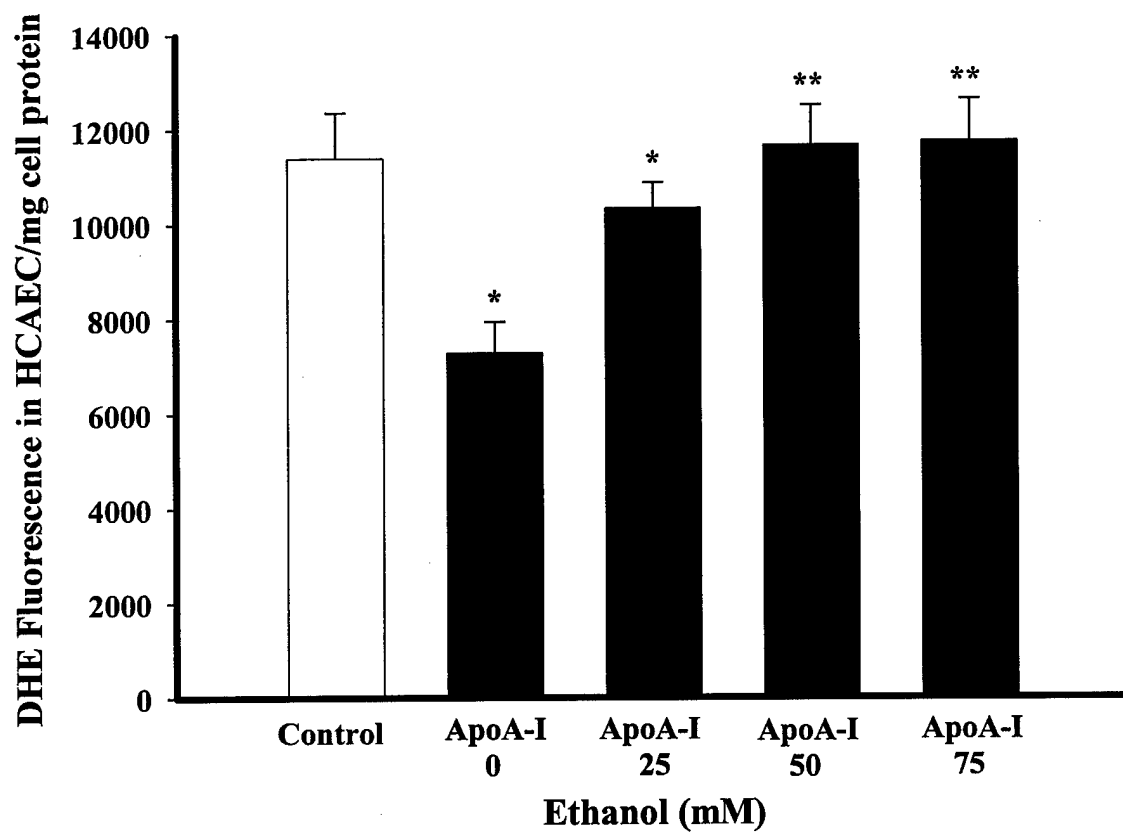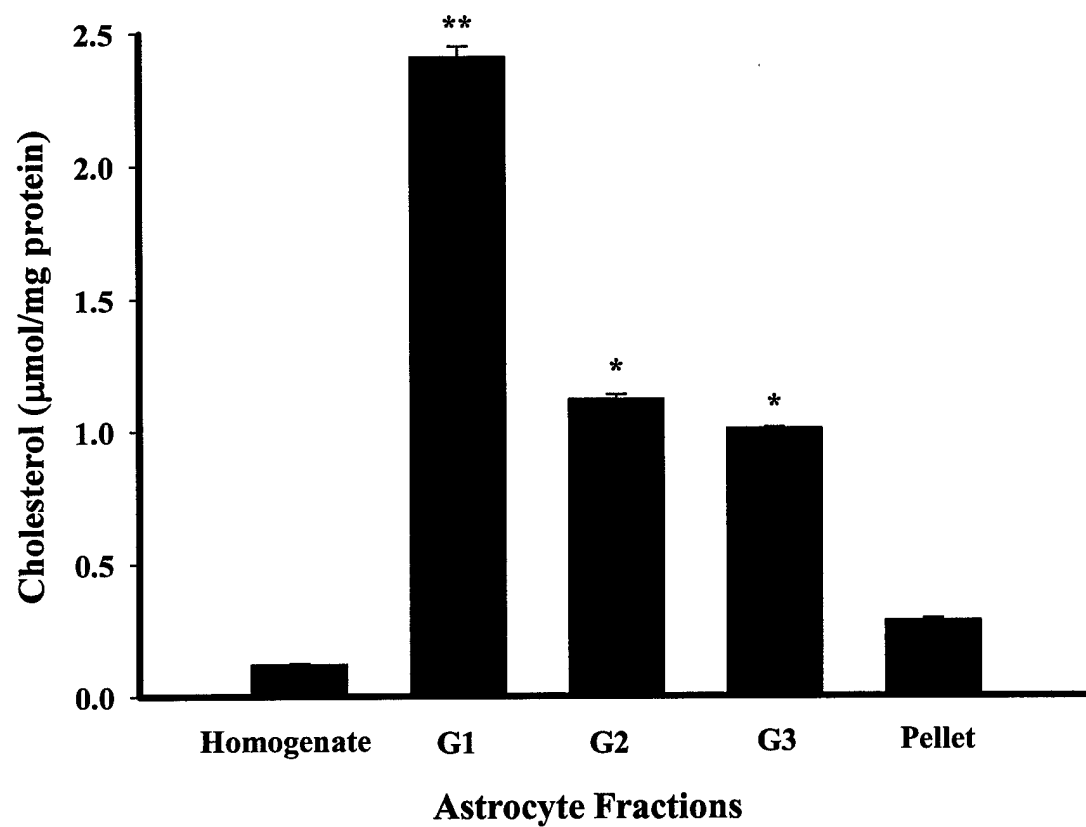
Figure 3. Ethanol Stimulates PC-PLD Activity in HASMC

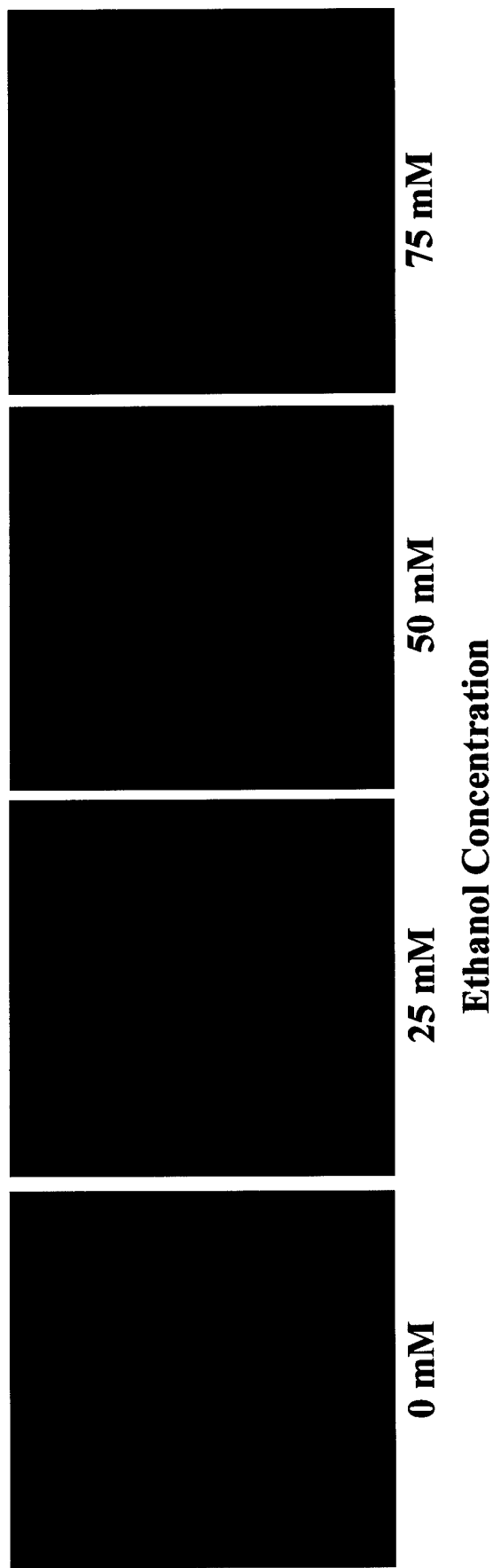# Figure 4. Effects of Ethanol on PC-PLC Activity in HASMC

Figure 5. Effects of Propranolol on ApoA-I Mediated Cholesterol Efflux in HASMC

Figure 6. Cholesterol Distribution in Golgi Complex and Different Fractions of Astrocytes

Ethanol Stimulates LDL Uptake in HCAEC Using BODIPY-LDL

| 0 mM | 25 mM | 50 mM | 75 mM |

Ethanol Concentration

Ethanol Stimulates LDL Uptake in HASMC Using DIL-LDL

| 0 mM | 25 mM | 50 mM | 75 mM |

Ethanol Concentration
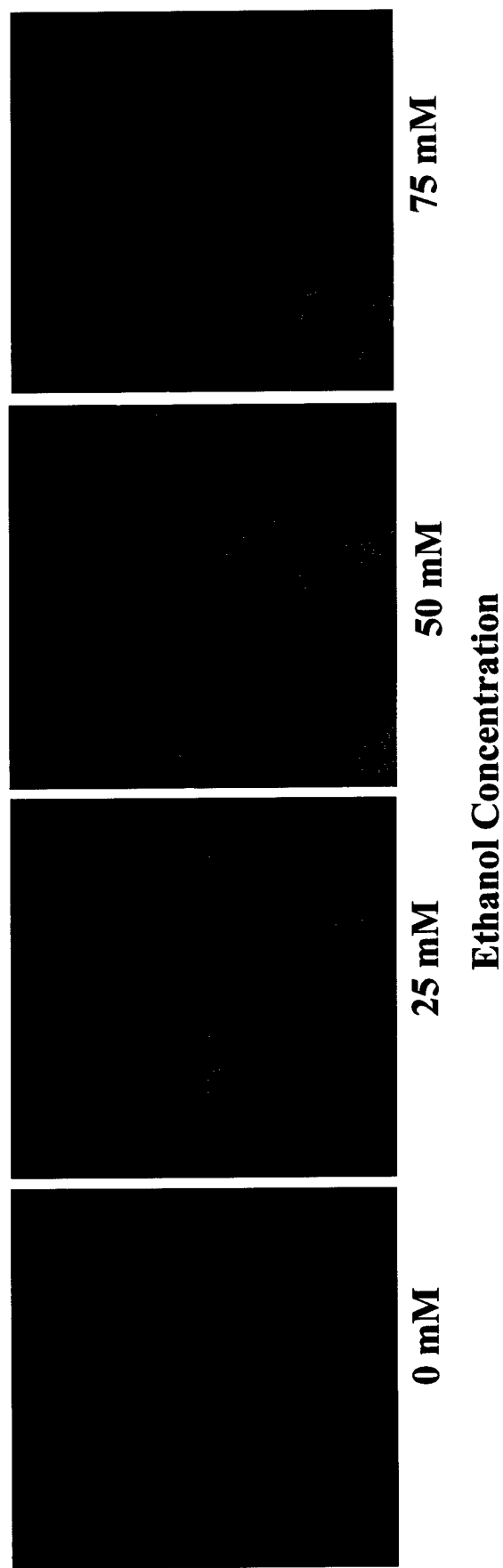
Figure 7

Figure 8. Ethanol Stimulates Uptake of LDL into HCAEC
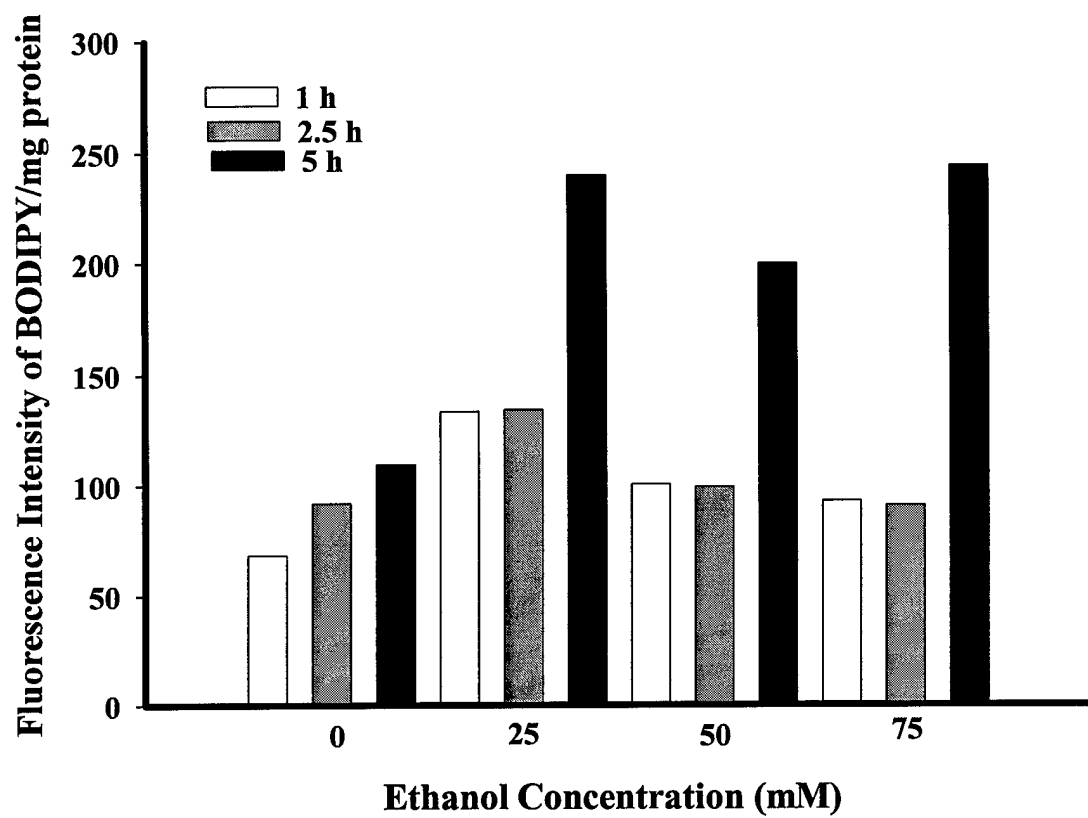
Legend:
- 1 h
- 2.5 h
- 5 h

Y-axis: Fluorescence Intensity of BODIPY/mg protein

X-axis: Ethanol Concentration (mM)

Figure 9. Ethanol Stimulates Uptake of LDL into HASMC

Legend:
- 1 h
- 2.5 h
- 5 h

Y-axis: Fluorescence Intensity of DIL/mg protein

X-axis: Ethanol Concentration (mM)
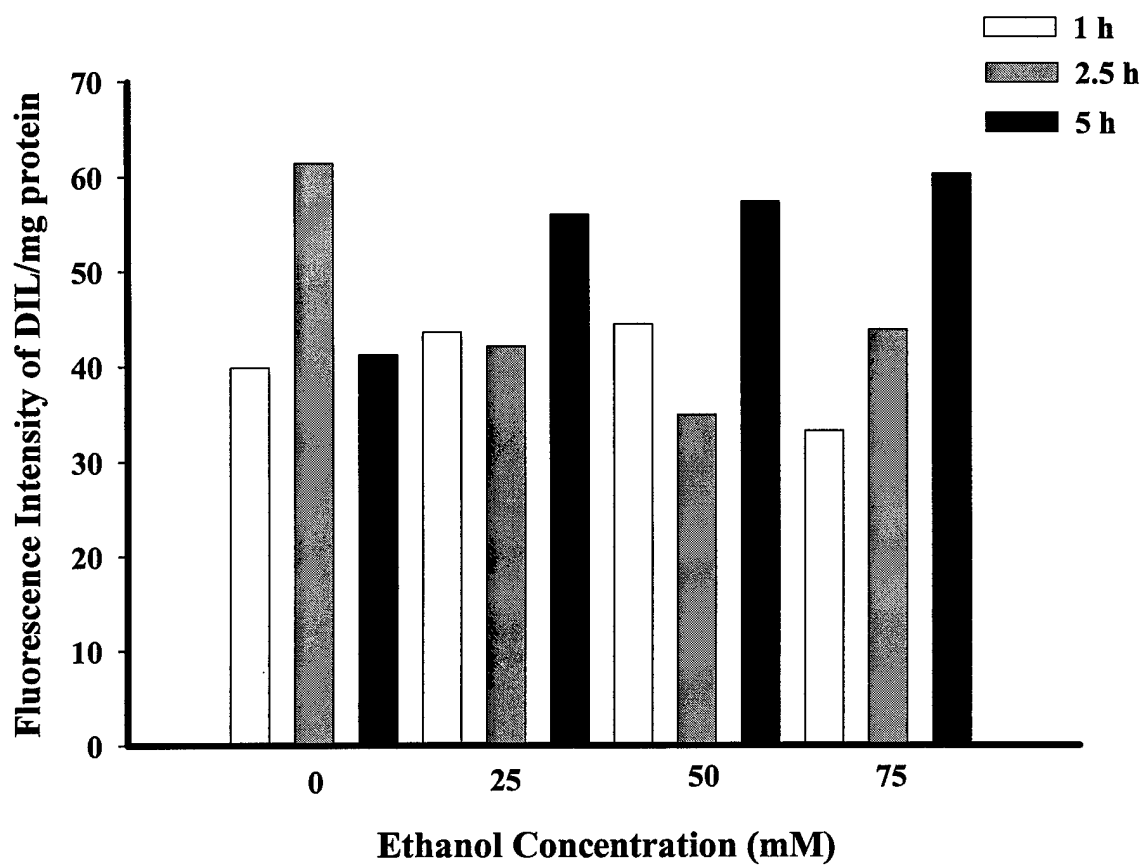
Alcoholism

CLINICAL AND EXPERIMENTAL RESEARCH

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Cholesterol Efflux is Inhibited by Ethanol in Human Aortic Smooth Muscle Cells:

Differences in Sensitivity of HDL and Apolipoprotein A-I and the

Roles of Phosphatidylcholine-Phospholipase C and D

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ABSTRACT

Background: Moderate alcohol consumption is associated with a reduced risk of cardiovascular morbidity and mortality. An increase in high-density lipoproteins (HDL) that are involved in cholesterol efflux are one of several factors that are thought to contribute to this reduced risk. However, HDL levels are elevated in heavy drinkers and alcoholics but their risk is not reduced. Ethanol may interfere with cholesterol efflux from cells and experiments in this paper report on acute effects of ethanol on cholesterol efflux from human aortic smooth muscle cells (HASMC) to HDL, apolipoprotein A-I (apoA-I) and the roles of phosphatidylcholine-phospholipase C (PC-PLC) and PC-phospholipase D (PC-PLD). Both PC-PLC and PC-PLD are involved in cholesterol efflux and also acted upon by ethanol. **Method:** Cholesterol efflux to HDL and apoA-I was determined in HSMAC using the fluorescent cholesterol analogue, dehydroergosterol. PC-PLC and PC-PLD activities were determined using an enzyme coupled assay. **Results:** Ethanol significantly inhibited HDL-mediated cholesterol efflux from HASMC. Significant effects of ethanol were seen at ethanol concentrations of 50 and 75 mM but not at 25 mM ethanol. ApoA-I was more effective in inducing sterol efflux than was HDL and significant inhibition occurred beginning at 25 mM ethanol. Ethanol stimulated activity of PC-PLD and PC-PLC but effects of ethanol were greater on PC-PLD. **Conclusions:** Ethanol may promote movement of cholesterol to the plasma membrane by stimulating phospholipase C and D-induced vesicular transport. However, removal of cholesterol from the plasma membrane may be inhibited by ethanol interfering with the physico-chemical interaction of HDL and particularly apoA-I with cell surface proteins. Heavy drinkers and alcoholics may be at risk with respect to acute and chronic effects of ethanol on cholesterol efflux and conversion of lipid-free apoA-I to mature HDL.

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Running title: Ethanol Inhibits Cholesterol Efflux in Human Aortic Cells

INTRODUCTION

Risk of coronary heart disease (CHD) morbidity and mortality is reduced by moderate alcohol consumption whereas some studies suggest that heavy alcohol consumption has little or no benefit for CHD (Eagles and Martin, 1998; Klatsky, 1999; Rimm, et al., 1999; Sesso and Gaziano, 1999). A generally accepted finding of epidemiological and experimental studies is that high-density lipoproteins (HDL) levels are increased in association with alcohol consumption (Castelli, et al., 1977; Klatsky, 1994; Rimm, et al., 1999; Srivastava, et al., 1994). An increase in HDL has been proposed to be one of the potential factors involved in the protective effects of moderate alcohol consumption on CHD (Castelli, et al., 1977; Criqui, et al., 1987). HDL levels however, are increased in heavy drinkers but their risk of CHD is higher than that of moderate alcohol drinkers (Frohlich, 1996; Goldberg, et al., 1995; Hojnacki, 1994). An important function of HDL is removal of excess lipids including cholesterol from cells and evidence has shown that chronic and acute administration of ethanol alters lipoprotein-mediated cholesterol efflux. Cholesterol efflux was decreased in mouse macrophages incubated with HDL of human alcoholic subjects (Rao, et al., 2000). However, it was recently reported that cholesterol efflux was stimulated from Fu5AH rat hepatoma cells to plasma of human subjects who consumed 40 g of alcohol per day for 3 weeks (van der Gaag, et al., 2001). Acute administration of ethanol (350 mM) increased the rate of cholesterol efflux from erythrocytes to plasma (Daniels and Goldstein, 1982). Ethanol concentrations (25 and 50 mM) that are seen in alcoholics and heavy drinkers inhibited cholesterol efflux from rat fibroblasts to HDL and apoA-I (Avdulov, et al., 2000).

Removal of lipids from cells involves several different mechanisms (e.g., membrane receptors, lipoproteins, signaling pathways, Golgi complex) that could be targets of ethanol

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perturbation. Ethanol for example has been reported to have a greater effect on lipid incorporation into apoA-I as compared with HDL (Avdulov, et al., 2000). ApoA-I is thought to activate phosphatidylcholine-PLD (PC-PLD) and phosphatidylcholine-phospholipase C (PC-PLC) resulting in phospholipid hydrolysis and production of phosphatidic acid (PA) and diacylglycerol, respectively (Walter, et al., 1996). Both PC-PLC and PC-PLD appear to be two of the mechanisms involved in regulating cholesterol efflux from cells (Walter, et al., 1996). PC-PLD is of particular importance with respect to action of ethanol because in the presence of alcohols there is a transphosphatidylation reaction and a phosphatidylalcohol is formed and a reduction of PA production (Heller, 1978; Moehren, et al., 1994). PA has been shown to stimulate cholesterol efflux (Walter, et al., 1996). Ethanol may alter activity of PC-PLC and PC-PLD that could impact on cholesterol efflux. Activity of PC-PLC in hepatocytes and activity of PC-PLD in fibroblasts were enhanced by ethanol (Kiss and Anderson, 1990; Pittner and Fain, 1992). Butanol which has a membrane/buffer partition coefficient several orders of magnitude higher than ethanol, inhibited cholesterol efflux of human fibroblasts and reduced production of phosphatidic acid (Walter, et al., 1996). In the same study it was shown that inhibition of PC-PLC by D 609 reduced cholesterol efflux and it was concluded that both PC-PLD and PC-PLC play a role in cholesterol efflux (Walter, et al., 1996).

The purpose of the experiments reported in this paper were to determine if acute ethanol administration altered cholesterol efflux induced by HDL and apoA-I in cells that are directly involved in cardiovascular function. Human aortic smooth muscle cells (HASMC) were used in this study. ApoA-I has been proposed to interact with the ATP binding cassette transporter 1 (ABC1) to remove cholesterol in contrast to HDL and HASMC are enriched in ABC1 (Fielding, et al., 2000; Wang, et al., 2000). Cholesterol efflux mediated by HDL and apoA-I may differ in

response to ethanol. In addition, effects of ethanol on activation of PC-PLC and PC-PLD activity were determined in HASMC using an enzyme coupled assay and *N*-acetyl-3,7-dihydroxyyphenoxazine (Zhou, et al., 1997).

METHODS

Materials. ApoA-I and HDL were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Amplex Red phosphatidylcholine-specific phospholipase C (A-12218) and phospholipase D (A-12219) assays kits were obtained from Molecular Probes (Eugene, Oregon). Human aortic smooth muscle cells and cell growth media (Smooth Muscle Cell Basal Medium (SmBM®), growth factors, trypsin/EDTA , HEPES Buffer Saline Solution (HBBS) and Trypsin Neutralizing Solution (TNS) were all obtained from Bio∗Whittaker Company (Walkersville, MD). FBS and antimytotic were obtained from HyClone, (Logan,UT). Lipoprotein deficient serum from human plasma was obtained from Sigma-Aldrich Chemicals (St. Louis, MO). 96-well plates for were purchased from USA Scientific, (Woodland, CA). L-Glutamine was obtained from Invitrogen:GibCo Corp. (Carlsbad, CA). Ethanol and all other reagents and chemicals were obtained from Sigma-Aldrich Chemicals (St. Louis, MO).

Cell Culture. Human aortic smooth muscle cells (HASMC) were grown in 25 cm^2 flask using SmBM® media and SMGM®- 2 Singlequot ®. The flasks with cells were maintained in an incubator at 37°C with 5% CO_2 . Media was replaced every 2 days. All experiments were performed with confluent cells.

Cholesterol Efflux. Confluent HASMC were preloaded with the fluorescent cholesterol analogue, dehydroergosterol (DHE) (3 μg/ml of cell culture medium) for 18 h in 0.5% lipoprotein deficient serum. DHE incubation time and concentration were determined from sampling incorporation of DHE into HASMC over 0-24 h. The optimal HDL or apoA-I concentration for our experiment was obtained by incubating the cells with different concentrations of HDL (10-40 μg of protein/ml of cell culture medium) or apoA-I for 2 h. HDL (30 μg of protein/ml of cell culture medium) was used in the cholesterol efflux experiments. In

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experiments using apoA-I, 30 µg of apoA-I/ml of cell culture medium were used and this amount is based on our recent report (Avdulov, et al., 2000). Cells were incubated for a period of 30 min with 0, 25, 50, or 75 mM ethanol after which 30 µg of human plasma HDL protein/ml of medium or 30 µg of apoA-I/ml of medium was added and cells were further incubated for 2 h. Cells were then washed twice with HBBS, trypsinized and neutralized with TNS or serum, collected, and centrifuged at 14,000 rpm for 1 min in an Eppendorf centrifuge (model 541 7R). The pellet containing cells was washed twice with 1 ml PBS and homogenized in a loose-fitting homogenizer. The protein of the homogenized samples was determined by Bradford method. Sample (25µg protein) was placed in a quartz cuvette and DHE fluorescence intensity determined using a LS-50B fluorimeter (Perkin-Elmer, Norwalk, CT) with the excitation wavelength of 324 nm and emission wavelength of 376 nm.

PC-PLC and PC-PLD Assays. PC-PLC activity and PC-PLD activity in HASMC were quantified using the Amplex red PC-PLC and PC-PLD assay kits (Molecular Probes, Eugene, OR). Cells were incubated with ethanol (0, 25, 50, and 75 mM) for 2 h after which time cells were then washed twice with HBBS, trypsinized and neutralized with TNS or serum, collected, and centrifuged at 14,000 rpm for 1 min in an Eppendorf centrifuge (model 541 7R). The pellet containing cells was washed twice with 1 ml PBS and homogenized in a loose-fitting homogenizer and aliquots placed in 96 well-plates and the assay reagents added. The intensity of the Amplex Red complex formed was measured after 60 min in a fluorescence microplate reader (Spectra Max Gemini XS, Molecular Device, Sunnyvale, CA) using an excitation wavelength of 542 nm and an emission wavelength of 590 nm.

RESULTS

Incorporation of Dehydroergosterol in HASMC. DHE is a sterol occurring in yeast, it is very similar to cholesterol in its structure and function, and it has been previously used as a cholesterol analogue in several different studies of sterol dynamics (reviewed in: Mukherjee, et al., 1998; Schroeder, et al., 1996). DHE is an ideal cholesterol analogue because the sterol is naturally fluorescent containing an endogenous fluorescent conjugated triene system in the B and C rings. DHE transport kinetics are analogous to those of cholesterol (Kier, et al., 1986; Mukherjee, 1998; Nemecz, et al., 1988). The purpose of these baseline experiments was to determine optimal conditions for DHE incorporation into HASMC. Three different concentrations of DHE (1,2,3 µg/ml of media) were incubated with HASMC for 18 h. Incorporation of DHE into cells was monitored by an increase in DHE fluorescence intensity. Figure 1 shows that DHE concentration of 3 µg/ml of media produced a robust increase in fluorescence intensity compared to the two lower concentrations. Fluorescence of non-incorporated DHE in the medium was negligible. The magnitude of fluorescence intensity was dependent also on the duration of incubation of DHE with cells. Fluorescence intensity of DHE steadily increased over time plateau ng between 18 and 24 h of incubation (Figure 2).

Sterol Efflux to HDL and ApoA-I. Figure 3 shows the effects of increasing concentrations of HDL on sterol efflux from HASMC. DHE fluorescence was measured in cells following removal of HDL by centrifugation. HDL significantly removed DHE from HASMC, and this effect was maximal at 30 µg of HDL protein/ml of medium and did not change significantly when 40 µg of HDL protein/ml of medium was added (Figure 3).

There was approximately a 20% reduction in cholesterol in cells incubated with HDL alone compared with no HDL and ethanol significantly inhibited HDL-mediated cholesterol

efflux from HASMC (Figure 4). Significant effects of ethanol were seen at ethanol concentrations of 50 and 75 mM ($p < 0.04$) but not at 25 mM ethanol (Figure 4). Effects of ethanol at 50 and 75 mM resulted in approximately 8-10% of cholesterol being removed from cells by HDL as compared to 20% removal by HDL alone.

There was a 24% reduction in sterol in cells when incubated with apoA-I alone as compared with cells not incubated with apoA-I (Figure 5). Ethanol significantly ($p \leq 0.001$) inhibited sterol efflux beginning at a concentration of 25 mM ethanol (Figure 5). In the presence of 50 and 75 mM ethanol, there was only approximately 6% and 3% of sterol removed respectively, by apoA-I.

PC-PLD and PC-PLC Activity. Both PC-PLC and PC-PLD have been proposed to be involved in cholesterol efflux. PC-PLD was significantly stimulated by ethanol (Figure 5). There was a 2-fold increase in PC-PLD activity by 25 mM ethanol as compared to control cells. It can be seen in Figure 5 that both 50 and 75 mM ethanol significantly ($p \leq 0.001$) increased PC-PLD activity as compared to control cells but this effect was not as large as observed for 25 mM ethanol.

Activity of PC-PLC was significantly ($p \leq 0.03$) increased by 25 mM ethanol as compared to the control cells resulting in a 16% increase in activity of the enzyme (Figure 6). PC-PLC activity was higher in the presence of 50 or 75 mM ethanol as compared to control but these differences were not significant.

DISCUSSION

Experiments in this paper tested the hypothesis that ethanol at concentrations observed in heavy drinkers and alcoholics would alter cholesterol efflux induced by HDL and apoA-I in human aortic smooth muscle cells. ApoA-I was used because recent evidence suggest that apoA-I may directly interact with cell surface receptors and that ethanol has a differential effect on apoA-I as compared with HDL (Fielding, et al., 2000; Avdulov, et al., 2000). In addition, effects of ethanol on PC-PLC activity and PC-PLD activity were examined. Both phospholipases have been previously shown to be involved in regulation of cholesterol efflux and acted upon by ethanol. We found that acute administration of ethanol inhibited cholesterol efflux from human aortic smooth muscle cells to HDL and apoA-I. Cholesterol efflux induced by apoA-I was more sensitive to effects of ethanol as compared with HDL. PC-PLD activity and PC-PLC activity were increased by ethanol with the greatest effect seen on PC-PLD activity.

Acute administration of ethanol inhibited cholesterol efflux induced by HDL and apoA-I from human aortic smooth muscle cells. ApoA-I had a greater effect on cholesterol efflux and was more sensitive to effects of ethanol compared with HDL. Significant inhibition of cholesterol efflux occurred at an ethanol concentration of 25 mM in the presence of apoA-I versus 50 mM in the presence of HDL. Differences in the magnitude of effects of ethanol on HDL and apoA-I may be due to direct effects of ethanol on those structures and interaction with cell surface proteins. Previously it has been reported that ethanol inhibited incorporation of cholesterol into apoA-I-PC complexes but did not alter incorporation of cholesterol into HDL (Avdulov, et al., 2000). There is also evidence that lipid efflux to apoA-I involves proteins at the cell surface such as ABC-1 transporter and caveolin (Fielding, et al., 2000; Lawn, et al., 1999). Recently, it was reported that cholesterol efflux from HASMC to apoA-I was derived from

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membrane microdomains enriched in caveolin (Fielding, et al., 2002). Ethanol has been reported to decrease the level of caveolin in astrocytes (Megias, et al., 2000) that might interfere with lipid efflux.

PC-PLC and PC-PLD have been shown to contribute to the regulation of cholesterol efflux (Haidar, et al., 2001;Walter, et al., 1996). PC-PLC hydrolyzes PC producing diacylglycerol and phosphocholine and PC-PLD also hydrolyzes PC producing phosphatidic acid (PA) and choline. ApoA-I and a subspecies of HDL, HDL_3 are thought to activate these two phospholipases (Haidar, et al., 2001; Walter, et al., 1996) that in turn induces transport of cholesterol to the plasma membrane. In the presence of primary alcohols and PC-PLD a transphosphatidylation reaction occurs with production of a phosphatidylalcohol instead of PA. Cholesterol efflux has been reported to be inhibited by *n*-butanol in fibroblasts (Haidar, et al., 2001;Walter, et al., 1996). We found that ethanol increased activity of both PC-PLC and PC-PLD with the largest effect observed for PC-PLD. The increase in activities of PC-PLD and PC-PLC by ethanol are in agreement with earlier reports in studies of hepatocytes and fibroblasts (Kiss and Anderson, 1990;Pittner and Fain, 1992). An ethanol concentration of 25 mM had a greater effect on activity of both phospholipases as compared with 50 and 75 mM ethanol particularly with respect to PC-PLD. This concentration dependent effect of ethanol could be due to inhibition of upstream effectors of PC-PLD and PC-PLC such as protein kinase C, G-protein-coupled receptors, and apoA-I (Haidar, et al., 2001).

There is evidence that the Golgi complex is important in cholesterol efflux and that an isoform of PC-PLD may be associated with the Golgi complex (Heino, et al., 2000; Ktistakis, et al., 1995; Mendez, 1995; Siddhanata, et al., 2000). Ethanol may increase transport of cholesterol from the Golgi complex to the plasma membrane by stimulating activity of PC-PLD. It has been

shown in fibroblasts that 40 mM and 80 mM ethanol significantly reduced cholesterol content in the Golgi complex as compared to control cells (Igbavboa, et al., 2001). If cholesterol originating in the Golgi complex is transported to the plasma membrane, efflux of cholesterol from the plasma membrane may be hindered by ethanol-induced perturbation of apoA-I, HDL and cell surface proteins. However, it has been reported that 1-butanol (135 and 200 mM) inhibited release of nascent secretory vesicles of the Golgi complex that was attributed to a reduction of PA formation (Chen, et al., 1997; Siddhanata, et al., 2000). The Golgi complex structure was disassembled in the study using 200 mM butanol (Siddhanata, et al., 2000). 1-Butanol is more hydrophobic than ethanol, the membrane/buffer partition coefficient of 1-butanol is markedly higher as compared with ethanol (1.52 versus 0.096), and higher 1-butanol concentrations were used in that study compared with the ethanol study that could contribute to differences between effects of ethanol and butanol on the Golgi complex.

HDL and apoA-I induced cholesterol efflux in HASMC was inhibited by ethanol. Ethanol has also been shown to have similar effects on cholesterol efflux in rat fibroblasts (Avdulov, et al., 2000). Conversely, cholesterol efflux was increased in rat hepatoma cells incubated with plasma from human subjects who had consumed ethanol (30-40 g etoh/day/for 2-3 weeks) (Senault, et al., 2000; van der Gaag, et al., 2001). Alcohol amounts used in those studies were considered moderate. On the other hand , HDL isolated from alcoholic patients were less efficient in removing cholesterol from mouse macrophages (Rao, et al., 2000). Ethanol has both an acute effect and a chronic effect on cholesterol efflux. In the present study, effects of acute administration of ethanol at amounts observed in heavy drinkers and alcoholics inhibited cholesterol efflux. A potential consequence of elevated blood alcohol levels is that conversion of lipid-free apoA-I to mature HDL may be hindered. Moderate alcohol consumption stimulated

cholesterol efflux whereas HDL of alcoholic patients were less efficient in removing cholesterol from cells. Heavy drinkers and alcoholics may be at greater risk for vascular disease as a result of both the acute and chronic effects of ethanol on regulation of cholesterol in cells.

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FIGURE LEGENDS

Figure 1: Incorporation of DHE into HASMC. In all experiments, confluent cells were used. DHE (3 μg/mL of cell culture medium) was added to cells, and at each time point, cells were processed and the DHE fluorescence was measured as described in the Methods section. Data are means ± SEM (n = 3). (Inset) Confluent HASMC were incubated with different concentrations of DHE for 18 h. Cells were then harvested and processed, and DHE fluorescence was measured. Data are means ± SEM (n = 3).

Figure 2: HDL-mediated efflux of DHE from HASMC. Confluent cells were incubated with 3 μg of DHE/mL of cell culture medium for 18 h. Cells were then washed twice using DHE-free medium and incubated with increasing concentrations of human plasma HDL for 2 h. Cells were then harvested and processed and DHE fluorescence was measured. Data are means ± SEM (n = 3). * $p \leq 0.02$ as compared with no HDL.

Figure 3: Ethanol Inhibits HDL-Mediated DHE Efflux from HASMC. Confluent HASMC were incubated with 3 μg of DHE/mL of cell culture medium for 18 h. Cells were then washed twice using DHE-free medium and incubated for 30 min with 25, 50, or 75 mM ethanol or no ethanol, after which 30 μg of human plasma HDL protein/mL of medium was added and cells were incubated for 2 h. Cells were then harvested and processed, and DHE fluorescence was measured. The control cells contained DHE but were not incubated with HDL or ethanol. Data are means ± SEM (n = 4). * $p \leq 0.04$ compared with HDL and no ethanol; ** indicates $p \leq 0.0001$ compared with control.

Figure 4: Ethanol Inhibits ApoA-I Mediated DHE Efflux from HASMC. Confluent HASMC were incubated with 3μg of DHE/mL of cell culture medium for 18 h. Cells were then washed twice using DHE-free medium and incubated for 30 min with 25, 50, 0r 75 mM ethanol or no

ethanol, after which 30 µg of human plasma apoA-I /mL of medium was added and cells were incubated for 2 h. Cells were then harvested and processed, and DHE fluorescence was measured. The control cells contained DHE but were not incubated with apoA-I or ethanol. Data are means ± SEM (n = 3). $^*p \leq 0.001$ as compared to apoA-I no ethanol; $^{**}p \leq 0.0002$ as compared to control no apoA-I.

Figure 5: Effects of Ethanol on PC-PLD Activity in HASMC. Confluent cells were incubated with different concentrations of ethanol for 2 h after which cells were harvested as described in Methods section. 20 µg of cells were placed in 96 well plates and PC-PLD determined using the Amplex® red PC-specific PLD assay kit. Samples were incubated for 60 min at 37°C. Fluorescence was measured with a microplate reader using an excitation wavelength of 542 nm and emission wavelength of 590 nm. Data are means ± SEM (n = 4). $^*p \leq 0.004$ as compared to 0 ethanol; $^{**}p \leq 0.0001$ as compared to each condition.

Figure 6: Effects of Ethanol on PC-PLC Activity in HASMC. Confluent cells were incubated with different concentrations of ethanol for 2 h after which cells were harvested as described in Methods section. 20 µg of cells were placed in 96 well plates and PC-PLC determined using the Amplex® red PC-specific PLC assay kit. Samples were incubated for 60 min at 37°C. Fluorescence was measured with a microplate reader using an excitation wavelength of 542 nm and emission wavelength of 590 nm. Data are means ± SEM (n = 4). $^*p \leq 0.02$ as compared to each condition.
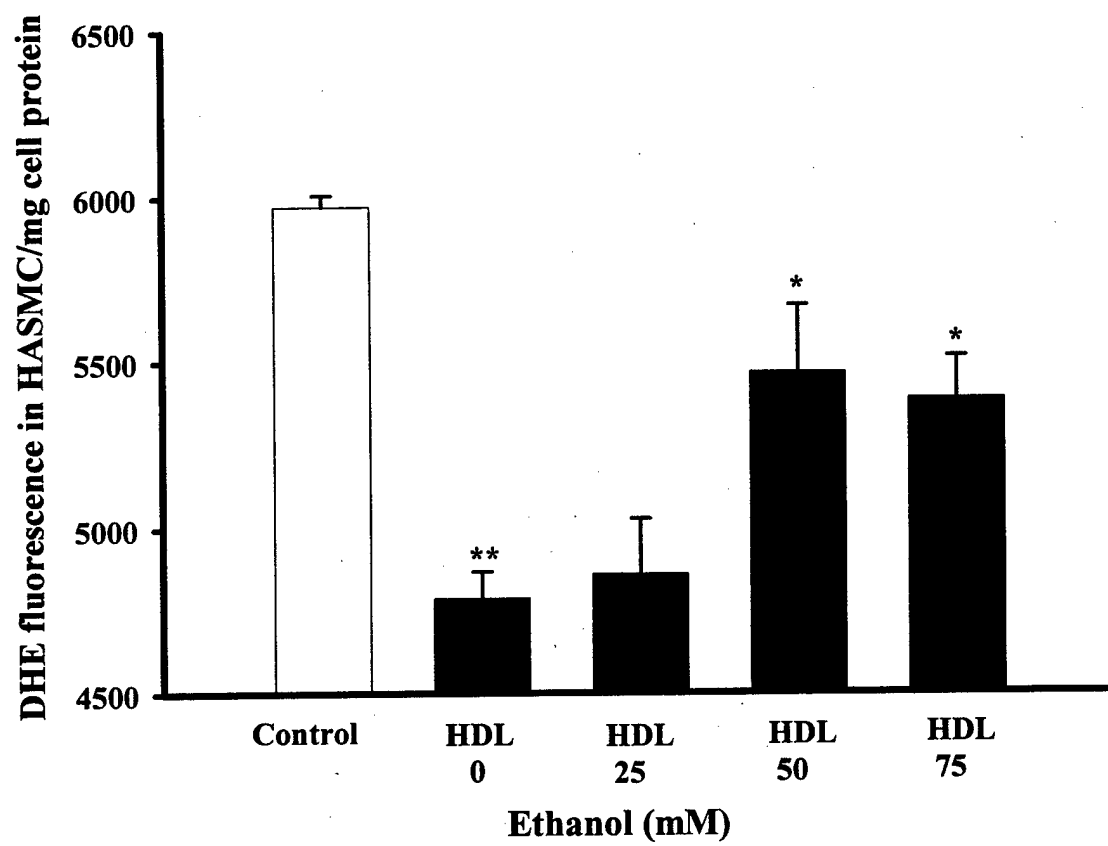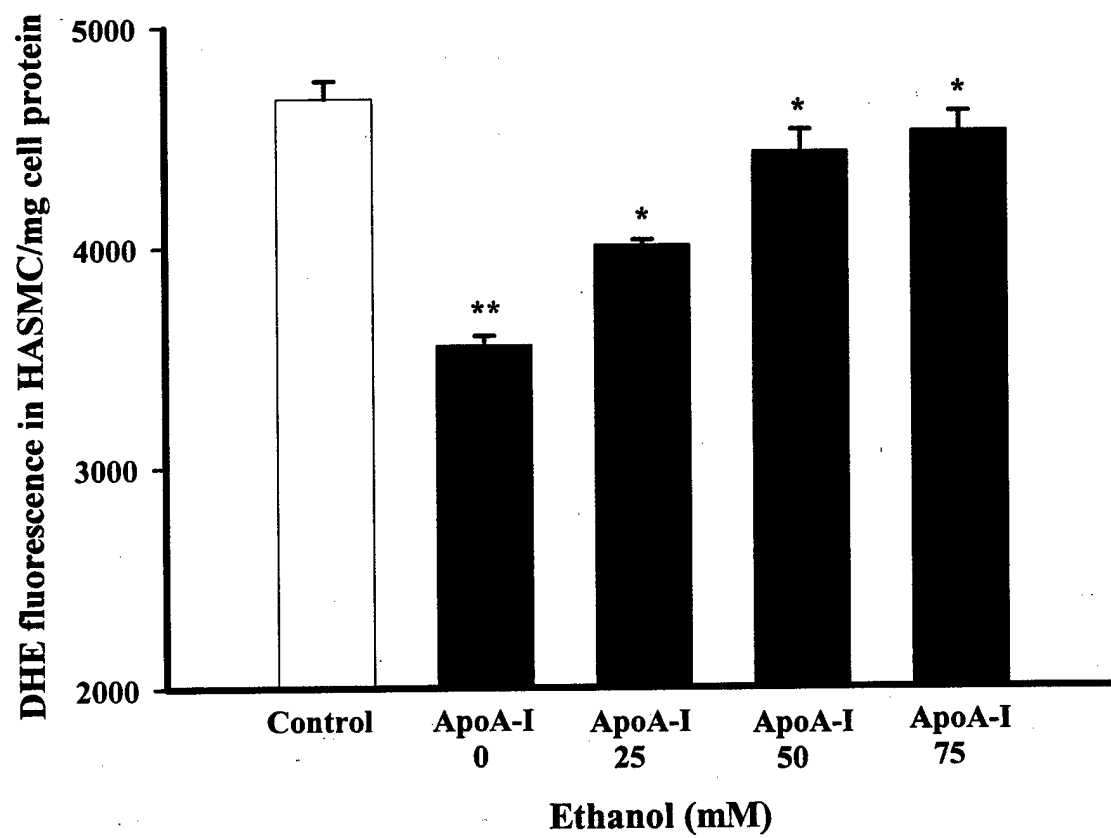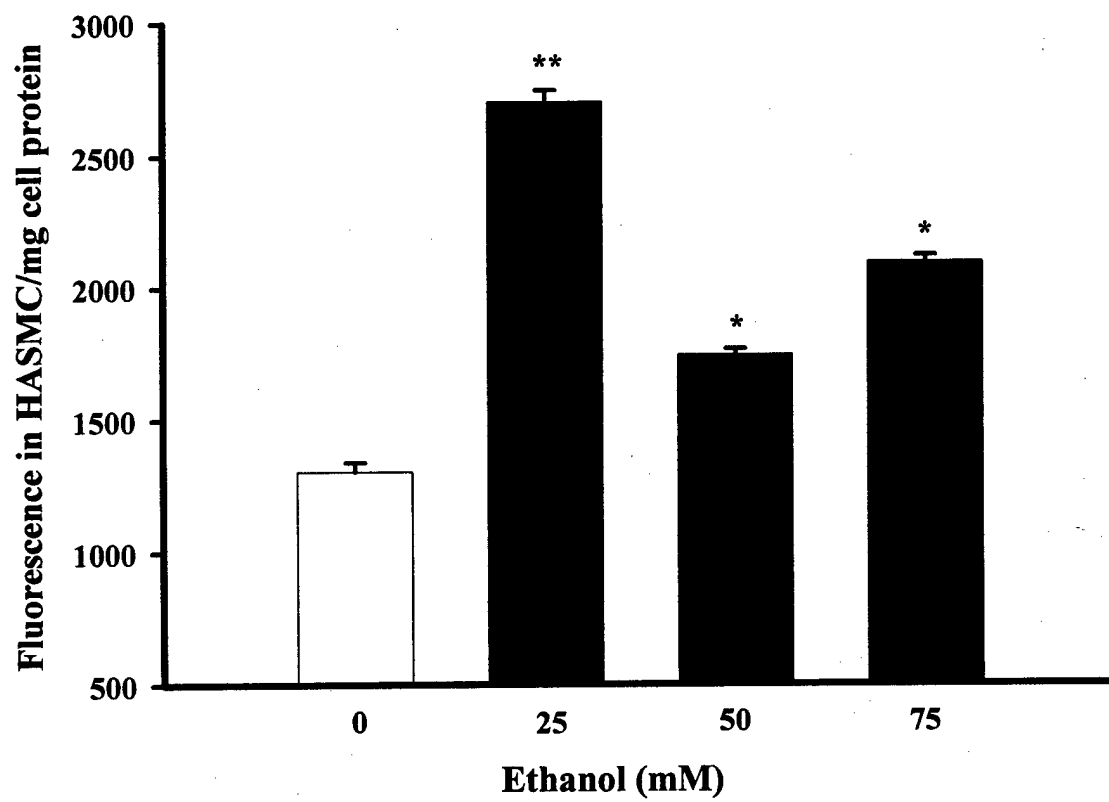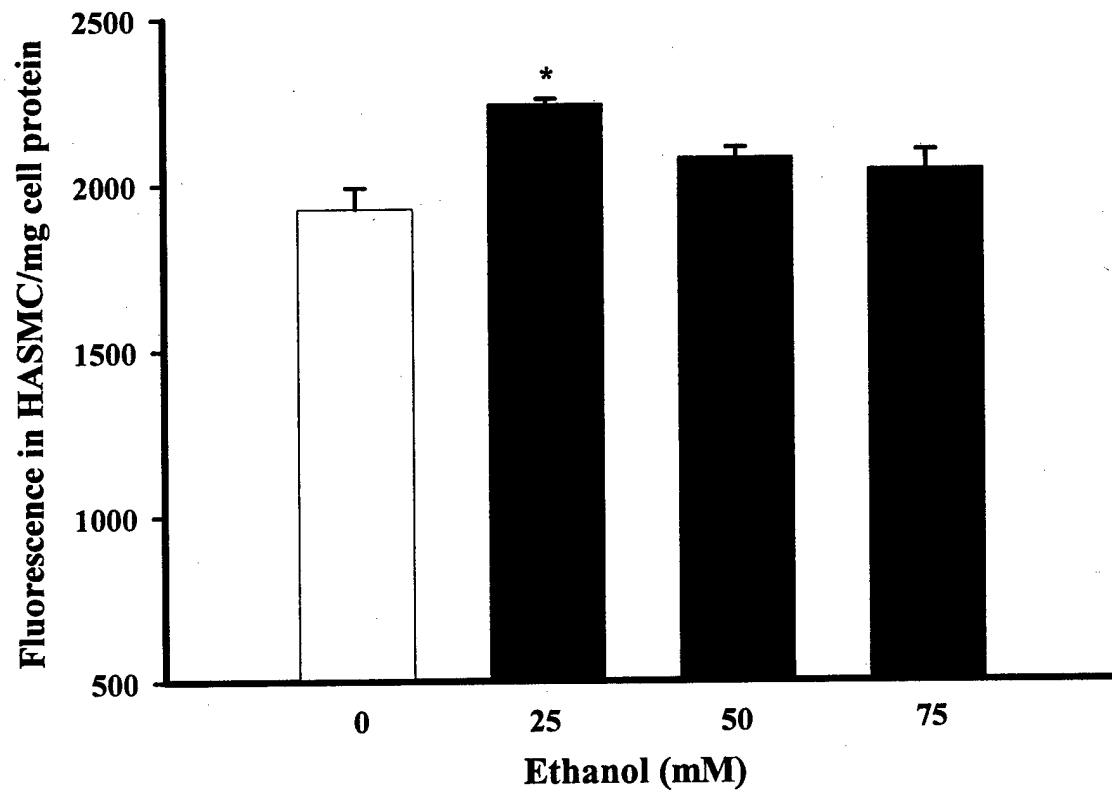
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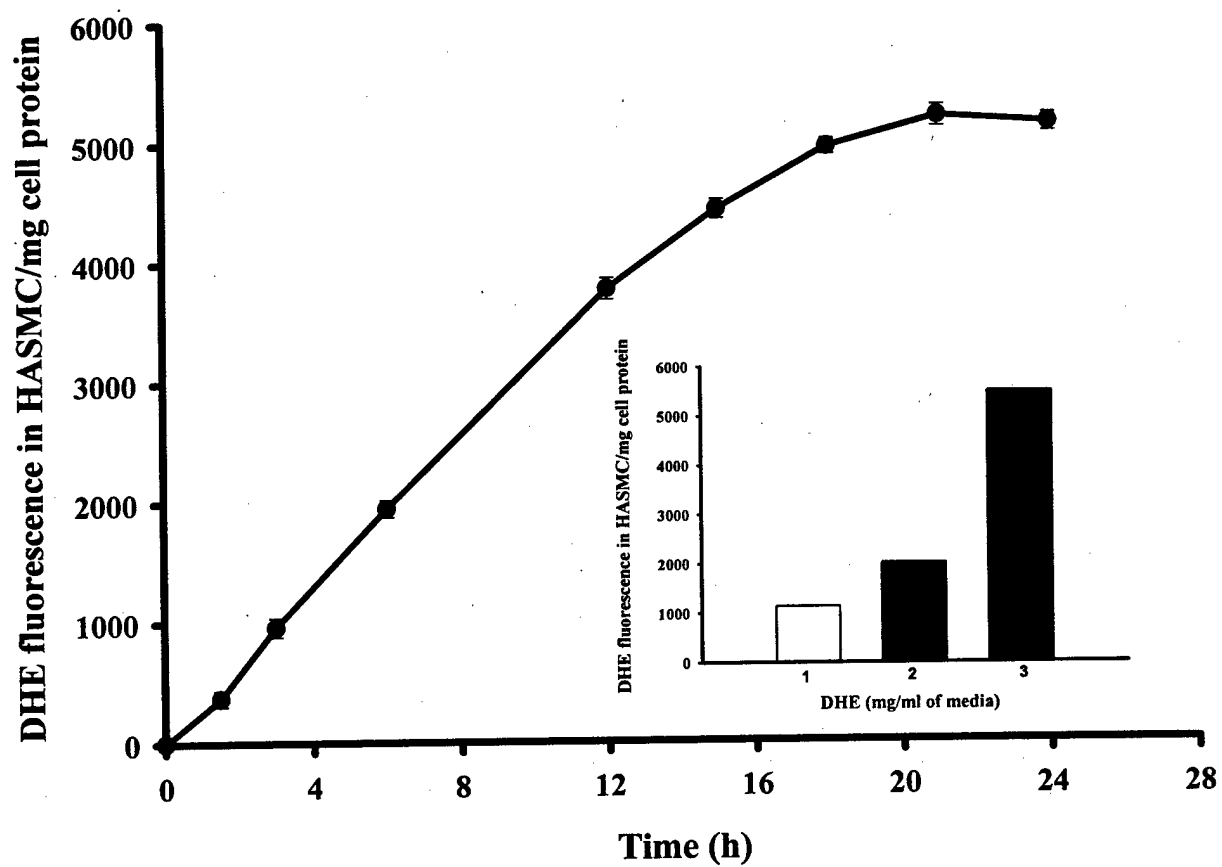
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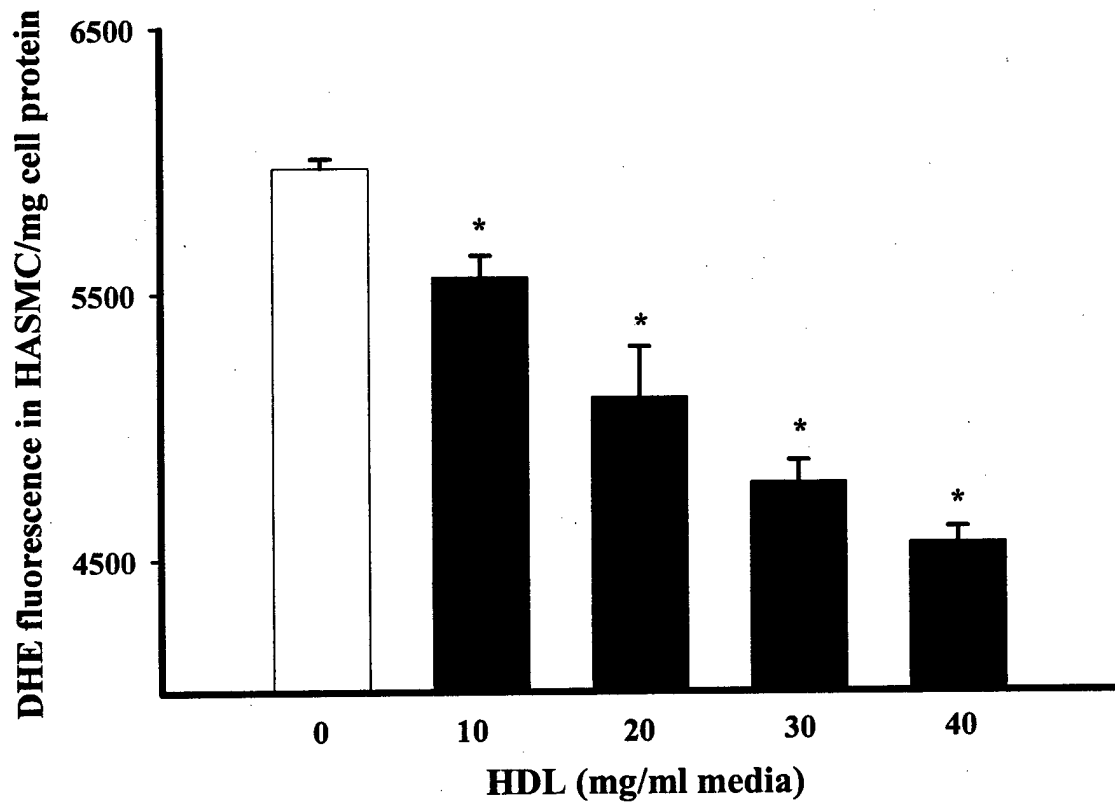
Figure 3

Figure 4

Figure 5

Figure 6

CHOLESTEROL DISTRIBUTION IN THE GOLGI COMPLEX OF ASTROCYTES IS ALTERED BY AMYLOID BETA-PEPTIDE$_{1-42}$: ROLES OF PEPTIDE STRUCTURE AND PHOSPHATIDYLCHOLINE-PHOSPHOLIPASE D

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Running Title: Amyloid beta-peptide alters cholesterol in Golgi complex

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Abstract

Amyloid beta-peptides are thought to play an important role in cellular pathophysiology associated with Alzheimer's disease (AD). Several emerging lines of evidence point to an interaction of amyloid beta-peptides and cholesterol dynamics. The Golgi complex has a central role in cholesterol trafficking, including cholesterol efflux from cells, and it has been shown that amyloid beta-peptide (Aβ) alters cholesterol efflux. We tested the hypothesis that soluble and oligomeric Aβ would differentially modify Golgi cholesterol content in astrocytes. In addition, phosphatidylcholine-phospholipase D has been proposed to be involved in regulation of cholesterol efflux, an isoform is associated with the Golgi complex. Activity of this phospholipase could be altered by Aβ and was examined. Two different methods were used to determine effects of Aβ on cholesterol in the Golgi complex: 1) confocal microscopy using the fluorescent cholesterol analogue, NBD-cholesterol and a fluorescent marker for the Golgi complex, BODIPY TR ceramide; and 2) isolation of two Golgi fractions using density gradient centrifugation. Effects of Aβ on Golgi complex cholesterol content were dependent on Aβ structure. Soluble Aβ significantly increased cholesterol and oligomeric Aβ significantly reduced cholesterol content in the Golgi complex as revealed by confocal microscopy. Isolation of the Golgi complex into two fractions by density gradient centrifugation showed similar effects of oligomeric Aβ as demonstrated by confocal microscopy but revealed the novel finding that soluble Aβ had opposite effects on the two Golgi fractions suggesting a specificity of Aβ perturbation on the Golgi complex. PC-PLD activity was inhibited by Aβ. Aβ-induced modification of Golgi cholesterol content could impact on important Golgi functions such as protein sorting, sphingomyelin synthesis and assembling and release of lipid rafts that could disrupt cell function and membrane structure.

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Introduction

Several different lines of evidence point to a potentially important but not well-understood interaction between Alzheimer's disease (AD) and cholesterol (reviewed in 1-4). Apolipoprotein E4, a protein that binds and transports cholesterol and other lipids, has been identified as a risk factor for familial and sporadic AD (5,6). Patients on inhibitors of HMG-CoA reductase (statins) , lovastatin and pravastatin have a lower risk of developing AD as compared with individuals not taking statins (7,8). A metabolite of brain cholesterol, 24S-hydroxycholesterol, was elevated in CSF of AD patients compared with control subjects (9).

Amyloid β -peptide (Aβ), the main component of neuritic plaques seen in brains of AD patients interacts with cholesterol. This interaction is reciprocal. Cholesterol levels modulate amyloid precursor protein (APP) and Aβ synthesis (10-13). Conversely, Aβ alters cholesterol dynamics. Aβ has a significantly greater effect on fluidity of synaptic plasma membranes (SPM) enriched in cholesterol as compared with SPM containing less cholesterol (14). Aβ increased the internalization of apoE complexed with cholesterol into neurons (15). Cholesterol efflux from rat hippocampal neurons to cyclodextrin was enhanced by Aβ and these results were attributed to redistribution of cholesterol to the plasma membrane (16).

The Golgi complex plays an important role in cholesterol trafficking (17,18) and Aβ could affect this process. Furthermore, effects of Aβ on cholesterol in the Golgi complex may be dependent on Aβ structure. Soluble Aβ was reported to be located in the hydrophobic area of SPM whereas oligomeric Aβ was intercalated adjacent to the phospholipid polar headgroup region (19). Recently, it was reported that oligomeric Aβ but not monomeric Aβ stimulated release of cholesterol, phospholipids and GM1 ganglioside from neurons (20). Oligomeric Aβ but not

soluble Aβ preferentially binds cholesterol as compared with fatty acids or phosphatidylcholine (21) that might enhance removal of lipids from cell compartments.

The current study tested the hypotheses that Aβ modifies Golgi complex cholesterol homeostasis and that effects of Aβ were dependent on peptide structure. In addition, it has been shown that phosphatidylcholine-phospholipase D (PC-PLD) contributes to regulation of cholesterol efflux and that an isoform of PC-PLD may be associated with the Golgi complex (17,18,22,23). Aβ could alter activity of PC-PLD and this possible action of Aβ was examined. Experiments were conducted using soluble and oligomeric Aβ$_{1-42}$ in immortalized DITNC1 astrocytes (24). Two different methods were used to determine effects of Aβ on Golgi cholesterol content: 1) confocal microscopy using the fluorescent cholesterol analogue, NBD-cholesterol and a fluorescent marker for Golgi, BODIPY TR ceramide (25); and 2) isolation of the Golgi complex into two different fractions thought to represent different regions of the Golgi complex using sucrose density gradient centrifugation (26,27). PC-PLD activity was determined in astrocytes using an enzyme coupled assay and N-acetyl-3,7-dihydroxyyphenoxazine (28). The results presented herein provide new insights into interaction of Aβ and cholesterol. We show that soluble Aβ$_{1-42}$ has a strikingly different effect on cholesterol content in the Golgi complex of astrocytes as compared with oligomeric Aβ$_{1-42}$. Incubation of astrocytes with soluble Aβ$_{1-42}$ significantly increased cholesterol whereas oligomeric Aβ$_{1-42}$ significantly reduced cholesterol content in the Golgi complex as shown by fluorescent probes and confocal microscopy. Similar findings were observed when the Golgi complex was isolated by sucrose density centrifugation. However, isolation of the Golgi complex into fractions thought to represent different parts of the Golgi complex revealed the novel finding that effects of soluble Aβ$_{1-42}$ on cholesterol content had opposite effects on the two Golgi fractions. The finding that PC-PLD activity was inhibited by

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soluble $A\beta_{1-42}$ was consistent with the increase in cholesterol content in the Golgi complex after treatment with soluble $A\beta_{1-42}$. Oligomeric $A\beta_{1-42}$ also inhibited PC-PLD activity but cholesterol content in the Golgi complex was reduced by oligomeric $A\beta_{1-42}$.

EXPERIMENTAL PROCEDURES

Materials. NBD-cholesterol (22-(N-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3 –β-ol) and BODIPY TR ceramide and Amplex Red phosphatidylcholine-specific phospholipase D (A-12219) assay kit were obtained from Molecular Probes (Eugene, Oregon). $A\beta_{1-42}$ was purchased from California Peptide Research Inc. (Napa, CA). Fetal Bovine Serum was obtained from HyClone (Logan, Utah). UDP[6-^{3}H] galactose was purchased from Amersham Biosciences (Piscataway, NJ). All other chemicals unless specifically mentioned were purchased from Sigma-Aldrich Chemical Company (St Louis, MO).

Cell Culture. DITNC1 rat astrocytes were purchased from American Type Culture Collection (Rockville, MD). These cells have been shown to have the phenotypic characteristics of type 1 astrocytes (24). All experiments were done using confluent astrocytes.

Incubation of Cells with Fluorescent Labeled Probes and $A\beta_{1-42}$. Astrocytes were grown on Lab-Tek™ chamber slides and incubated with NBD-cholesterol (8 µM) for 1 h and then washed 3 times with 1ml PBS. Soluble $A\beta_{1-42}$ (1 µM) or $A\beta_{1-42}$ (1 µM) pre-incubated for 48 h (oligomeric) was added to cells and incubated for 2 h. We have previously reported that incubation of $A\beta_{1-42}$ for 48 h resulted in approximately 74% of the peptide in a tetrameric form (19). The cells were rinsed three times with PBS and the Golgi marker, BODIPY TR ceramide (2 µM) was added and incubated for 1 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde and mounted for confocal microscopy using Gel/Mount from Biomeda Corp. (Foster City,CA).

Laser Scanning Confocal Microscopy. Confocal fluorescence imaging was performed on an Olympus Fluoview Laser Scanning Confocal Imaging System (Olympus America Inc., New York, NY). Images were captured using multiple photomultiplier tubes regulated by Fluoview

2.0 software (Olympus). Excitation of the fluorescence probes was accomplished using 15 mW

krypton-argon lasers with 5 mW output. An Olympus BX 50 fluorescent microscope was used

to capture the images using an oil-immersion objectives (60X astrocytes; 100X neurons). NBD-

cholesterol was excited at 488 nm and emission was recorded at 540 nm. BODIPY TR ceramide

was excited at 568 nm and emission recorded at 598 nm. The captured images for the red and

green channels were merged and appeared yellow indicative of colocalization. Red and green are

additive, generating yellow to orange in RGB color space (29). Quantitative analysis of the

colocalization of NBD-cholesterol and BODIPY TR ceramide was determined by image

processing using MetaMorph Imaging system V4.3 from Universal Imaging Corp. (West

Chester, PA) as previously described (29,30) and expressed as percent colocalization.

Isolation of Golgi Fractions. Isolation of the Golgi complex was accomplished using sucrose

density gradient centrifugation (27). Confluent astrocytes were treated with soluble and

oligomeric $A\beta_{1-42}$ as described above. Cells were harvested and washed twice with PBS. The

cells were suspended in G-buffer (10 mM Tris-HCl, 0.25 M Sucrose and 2 mM $MgCl_2$, pH 7.4)

containing 10 mM $CaCl_2$. Leupeptin and PMSF were added to the cell suspension to inhibit

proteolytic enzymes, prior to homogenization. The cells were homogenized in a Potter-type

homogenizer and homogenized with 20-30 strokes using a serrated homogenizing pestle. The

homogenate was centrifuged at 5,000 x g for 10 min. The pellet was suspended and

homogenized in 1.4 M sucrose and overlayered with 0.8 M, 1.0 M, 1.2 M sucrose gradients in

ultracentrifuge tubes. Samples were then centrifuged at 95,000 x g for 2.5 h in a SW28 rotor in a

Beckman L8-70 ultracentrifuge. Each interface was carefully removed and diluted with G buffer

and centrifuged in a SS34 rotor at 45,900 x g for 20 min. Each pellet was suspended in buffer

and used for the experiments. Protein concentrations of the fractions were determined using

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Bradford assay. Cholesterol content in each fraction was determined enzymatically in a microassay using the Boehringer-Mannheim diagnostic kit (31) and procedures reported by our laboratory (32-34).

Galactosyltransferase Activity. A marker enzyme of the Golgi complex is galactosyltransferase and activity of this enzyme was measured using procedures previously reported (27,35). The incubation mixture contained 7mg/ml ovalbumin, 2 mM ATP, 200 mM $MgCl_2$, 0.2% Triton-X-100, 50 mM Tris-HCl (pH 6.8). The reaction was initiated by adding [^{3}H]UDP-galactose to samples, incubated for 30 min and the reaction was stopped by adding ice-cold 24% trichloroacetic acid (TCA). The precipitate was pelleted at 20,800 x g and washed three times with 12% TCA. Sample was solubilized in 5% SDS and dpm counted using a scintillation counter.

PC-PLD Activity. PC-PLD activity of control astrocytes and astrocytes that had been incubated with either soluble or oligomeric $A\beta1_{-42}$ for different time periods was quantified using the Amplex red PC-PLD assay kit (Molecular Probes, Eugene, OR). The intensity of the Amplex Red complex formed was measured after 60 min in a fluorescence microplate reader (Spectra Max Gemini XS, Molecular Device, Sunnyvale, CA) using an excitation wavelength of 542 nm and an emission wavelength of 590 nm.

RESULTS

Aβ$_{1-42}$ Modifies Cholesterol Distribution in the Golgi Complex as Revealed by Confocal Microscopy. This study tested the hypothesis that Aβ$_{1-42}$ would modify cholesterol content in the Golgi complex of astrocytes. It was further proposed that effects of Aβ on cholesterol distribution would be dependent on Aβ structure and PC-PLD activity. Two different methods were used to determine cholesterol distribution in the Golgi complex: 1) A fluorescent cholesterol analogue, NBD-cholesterol and a fluorescent marker for the Golgi complex, BIODPY TR ceramide were used and imaged with confocal microscopy; and 2) sucrose density gradient centrifugation was used to isolate two different fractions of the Golgi complex.

BODIPY TR ceramide, NBD-cholesterol and confocal microscopy were used to initially examine effects of soluble and oligomeric Aβ$_{1-42}$ on Golgi cholesterol content of astrocytes. Confocal images of colocalization of NBD-cholesterol and BODIPY TR ceramide in control and Aβ treated astrocytes can be seen in Figure 1. Panel C shows colocalization of the two fluorescent probes in control astrocytes. It can be seen in panel F that colocalization was greater in astrocytes treated with soluble Aβ whereas, oligomeric Aβ (panel I) reduced colocalization as compared with control astrocytes. Analysis of colocalization data using MetaMorph software and expressed as percent colocalization shows that soluble Aβ$_{1-42}$ significantly ($p \leq 0.001$) increased colocalization in the Golgi complex of astrocytes (Figure 2) as compared with control astrocytes. The percent colocalization of the fluorescent probes was significantly ($p \leq 0.01$) less when astrocytes were incubated with oligomeric Aβ$_{1-42}$ as compared with control astrocytes (Figure 2).

UDP-Galactosyltransferase Activity and Fluorescence of BODIPY TR-Ceramide in Golgi Complex Fractions. Confocal microscopy revealed that Aβ$_{1-42}$ altered the colocalization of NBD-cholesterol and BODIPY TR ceramide, a marker for the Golgi complex, and these data

were interpreted as $A\beta_{1-42}$ altering Golgi cholesterol content. The Golgi complex is heterogenous in structure and function and may not be equally affected by $A\beta$. To further define effects of soluble and oligomeric $A\beta_{1-42}$ on cholesterol distribution in the Golgi complex of astrocytes, the Golgi complex was isolated using sucrose density centrifugation and two different fractions were obtained that are thought to represent different regions of the Golgi complex (27). Enrichment of UDP-galactosyltransferase activity and fluorescence of BODIPY TR ceramide have been previously used as markers for the Golgi complex (25,27,36,37) and these markers were used in the present study. Figure 3 shows UDP- galactosyltransferase activity in homogenate, the light Golgi fraction (G1) which is the band at the 0.8-1.0 sucrose gradient interface and the Golgi fraction 2 (G2) which is the band at the 1.0-1.2 sucrose gradient interface. UDP-galactosyltransferase activity was significantly higher ($p < 0.01$) in the G1 fraction as compared with the G2 fraction. UDP- galactosyltransferase activity was several orders of magnitude higher in both the G1 and G2 fractions in contrast to the homogenate (Figure 4). Enrichment of UDP-galactosyltransferase activity in the G1 fraction compared with the G2 fraction has been previously reported (27).

BODIPY TR ceramide, a fluorescent Golgi marker, had a similar distribution in the G1 and G2 astrocyte fractions as was observed for UDP-galactosyltransferase activity. It can be seen in Figure 4 that the fluorescence intensity of BODIPY TR ceramide was significantly higher ($p < 0.001$) in the G1 fraction than the G2 fraction. Fluorescence intensity of BODIPY TR ceramide was highly enriched in both fractions when compared with the homogenate (Figure 4).

$A\beta_{1-42}$ Modifies Cholesterol Distribution in the Golgi Complex Fractions as Revealed by Sucrose Density Centrifugation. Figure 5 shows that there was significantly ($p \le 0.001$) more cholesterol in the G1 fraction of control astrocytes in contrast to the G2 fraction. Similar findings were

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observed with NBD-cholesterol (data not shown). Effects of soluble and oligomeric $A\beta_{1-42}$ on cholesterol in the G1 fraction were similar to effects we observed using confocal microscopy. Soluble $A\beta_{1-42}$ significantly ($p < 0.0001$) increased cholesterol whereas oligomeric $A\beta_{1-42}$ significantly ($p < 0.001$) reduced cholesterol in the G1 fraction (Figure 5). It can be further seen in Figure 5 that the magnitude of effects of soluble $A\beta_{1-42}$ on cholesterol in the G1 fraction was greater than oligomeric $A\beta_{1-42}$ and these results were similar to those observed with confocal microscopy.

Figure 5 shows that soluble $A\beta_{1-42}$ had an opposite effect on cholesterol in the G2 fraction as compared with the G1 fraction. There was a significant ($p < 0.001$) reduction in cholesterol in the G2 fraction when incubated with soluble $A\beta_{1-42}$. On the other hand, oligomeric $A\beta_{1-42}$ had a similar effect on cholesterol in the G2 fraction as observed in the G1 fraction, resulting in a significant ($p < 0.001$) reduction in cholesterol (Figure 5).

$A\beta_{1-42}$ induced modification of cholesterol in the Golgi complex of astrocytes was not attributable to changes in cholesterol metabolism. In can be seen in Figure 5 that the total amount of cholesterol in the homogenate fraction was not altered by either soluble or oligomeric $A\beta_{1-42}$ when compared with control astrocytes.

$A\beta_{1-42}$ Inhibits PC-PLD Activity. PC-PLD has been shown to be involved in regulating cholesterol efflux from cells (38) and an isoform of PC-PLD has been reported to be associated with the Golgi complex (17;22). Effects of soluble and oligomeric $A\beta_{1-42}$ on activity of PC-PLD were examined in astrocytes that had been incubated with $A\beta$ for different time periods. Figure 6 (panels A & B) reveals that soluble and oligomeric $A\beta_{1-42}$ significantly ($p < 0.001$) inhibited PC-PLD activity at each incubation time period. There was some diminution of effects of $A\beta$ on PC-PLD activity with increasing incubation time. PC-PLD activity was significantly ($p < 0.001$)

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higher after 120 min of incubation versus 30 min of incubation for both soluble and oligomeric

$A\beta_{1-42}$ (Figure 6A & B).

DISCUSSION

Several different lines of evidence show that there is an interaction between $A\beta$ and cholesterol (2,3). This interaction is reciprocal given that cholesterol modulates expression of APP and $A\beta$ and cholesterol dynamics such as cholesterol trafficking are affected by $A\beta$. The Golgi complex plays an important role in cholesterol trafficking and we proposed that $A\beta$ would alter cholesterol trafficking in the Golgi complex and effects of $A\beta$ would be dependent on peptide structure. In addition, effects of $A\beta$ on PC-PLD were determined. PC-PLD plays a role in cholesterol trafficking and an isoform is associated with the Golgi complex. Two different methods, confocal microscopy and isolation of the Golgi complex by sucrose density centrifugation were used to test the hypothesis that soluble and oligomeric $A\beta$ would have different effects on cholesterol in the Golgi complex of astrocytes. Results of this study showed that $A\beta_{1-42}$ modifies cholesterol distribution in the Golgi complex of astrocytes. However, effects of $A\beta$ on cholesterol distribution were dependent on $A\beta$ structure. Soluble $A\beta$ increased cholesterol content in the Golgi complex and oligomeric $A\beta$ reduced cholesterol content in the Golgi complex. Moreover, isolation of the Golgi complex into two fractions revealed the novel finding that soluble $A\beta$ increased cholesterol in the G1 fraction and reduced cholesterol in the G2 fraction. We also observed that activity of PC-PLD was inhibited by both soluble and oligomeric $A\beta_{1-42}$.

$A\beta_{1-40}$ has been shown to stimulate cholesterol efflux from neurons to cyclodextrin and it was suggested that $A\beta$ may alter vesicle transport (16). Recently, it was reported that oligomeric $A\beta_{1-40}$ stimulated lipid release including cholesterol from astrocytes and neurons (20). Monomeric and fibrillar $A\beta$ did not have an effect on lipid release. In the present study, we found that oligomeric $A\beta_{1-42}$ reduced cholesterol in the Golgi complex but that soluble $A\beta_{1-42}$ had

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an opposite effect. It is well-established that the Golgi complex is important in regulating cholesterol efflux and our findings indicate that the Golgi complex is a target of Aβ. Oligomeric Aβ may sequester lipids in the exofacial leaflet membranes and that in turn is a signal to the Golgi complex to transport cholesterol to the plasma membrane. These conclusions are based on the following lines of evidence. Oligomeric Aβ$_{1-40}$ removed lipids including cholesterol from astrocytes and neurons and formed Aβ-lipid complexes (20). It has been previously reported that oligomeric Aβ$_{1-40}$ binds cholesterol and other lipids whereas soluble Aβ$_{1-40}$ did not bind lipids (21). Oligomeric Aβ$_{1-40}$ was found to be positioned in SPM at the phospholipid polar headgroup/water interface, whereas soluble Aβ$_{1-40}$ intercalates deep into the hydrophobic membrane core (19). While it cannot be concluded that oligomeric Aβ does not partition into the cell, the membrane surface may be more energy favorable with respect to electrostatic interactions and binding (39-41). Removal of cholesterol from cells by oligomeric Aβ may stimulate recruitment of cholesterol from the Golgi complex to the plasma membrane. There is evidence that both the Golgi complex and PC-PLD have roles in cholesterol efflux and an isoform of PC-PLD may be associated with the Golgi complex and vesicular trafficking (17,18,22,23). Apolipoprotein A-I and HDL$_3$ can induce activity of PC-PLD by action of a G-protein-coupled receptor that results in hydrolysis of PC and the production of phosphatidic acid (PA) and choline. Inhibition of PC-PLD reduces cholesterol efflux whereas administration of PA stimulates cholesterol efflux (22,38). Activation of PC-PLD enhanced the release of secretory vesicles from the trans-Golgi network (42). Our findings that soluble Aβ increased cholesterol content in the Golgi complex and inhibited activity of PC-PLD are in agreement with the purported role of PC-PLD in cholesterol trafficking. However, oligomeric Aβ reduced cholesterol in the Golgi complex and inhibited activity of PC-PLD. Oligomeric Aβ may be

acting on a different cholesterol trafficking pathway as compared with soluble Aβ. Another potential pathway involves phosphatidylcholine-phospholipase C (PC-PLC) that also regulates cholesterol efflux. Hydrolysis of PC by PC-PLC produces diacylglycerol and phosphocholine (38). Diacylglycerol is an activator of protein kinase C (PKC) and there are some data suggesting PKC contributes to cholesterol efflux (43). Inhibition of PC-PLC by D609 reduced cholesterol efflux (38). It was shown that H7, an inhibitor of PKC, blocked the stimulatory effects of oligomeric Aβ_{1-40} on cholesterol efflux (20). Oligomeric Aβ may stimulate activity of PC-PLC resulting in an increase in diacylglycerol production and PKC-induced cholesterol efflux. Our findings that Aβ_{1-42} inhibited activity of PC-PLD differ from a study showing that the Aβ peptide fragment 25-35 increased PLD activity in LA-N-2 cells (44) and such differences between the two studies may have resulted from dissimilarities in peptide structure.

Soluble Aβ_{1-42} increased cholesterol in the G1 fraction but reduced cholesterol in the G2 fraction. Earlier work has indicated that the G1 fraction may represent the trans Golgi region and the G2 fraction is representative of the medial and cis regions (26,27). There are also data indicating that there is a gradient of cholesterol distribution with the trans Golgi region containing a higher level of cholesterol as compared with the medial and cis regions (45,46). Our data on cholesterol distribution showed that the G1 fraction had significantly more cholesterol than did the G2 fraction. This finding is similar to what we observed when the fluorescence intensity of NBD-cholesterol was examined in the two fractions (data not shown). Fluorescence intensity of NBD-cholesterol was significantly higher in the G1 fraction than the G2 fraction. Soluble Aβ may stimulate movement of cholesterol from the cis and medial regions of the Golgi to the trans Golgi region but the increase in cholesterol in the G1 fraction cannot be entirely accounted for by such a mechanism. Aβ_{1-40} and Aβ_{1-42} have been reported to inhibit cholesterol

esterification in human plasma and rat cortical neurons from primary cultures, respectively (16,47). In the study using neurons, inhibition of cholesterol esterification by Aβ was associated with an increase in free cholesterol levels (16). A portion of the increased cholesterol could be transported to the Golgi. Cholesterol esterification in cells occurs primarily in the endoplasmic reticulum and unesterified cholesterol can be transported to the Golgi complex. Another potential mechanism may involve transport of cholesterol from the plasma membrane to the Golgi complex. There is evidence that transport of sphingolipids occurs from the plasma membrane to the Golgi complex by two independent endocytic pathways and that some cholesterol may also be transported to the Golgi complex (48). Soluble Aβ that easily partitions into the plasma membrane in contrast to oligomeric Aβ could act to stimulate these pathways that might result in an increase in cholesterol content in the trans Golgi region and possibly sphingolipids.

The Golgi complex has been described as a major site for protein and lipid sorting (45). Aβ-induced changes in the cholesterol content of the Golgi complex could have several potential pathophysiological consequences. Indeed, there is evidence showing that that the Golgi complex generates different Aβ species (49). Cholesterol transport from the Golgi complex to the plasma membrane could be altered that in turn could modify membrane structure and function. Soluble Aβ may inhibit formation of lipid rafts in the Golgi complex including caveolin organized rafts that are transported to the plasma membrane and form caveolae. The amyloid precursor protein (APP) colocalizes with caveolin-3 (50) and APP and Aβ peptides are located in detergent insoluble membrane domains that are thought to be caveolae (51,52) and cholesterol domains not containing caveolin (53).

This paper showed that Aβ altered cholesterol content of the Golgi complex of astrocytes. Effects of Aβ on cholesterol content were dependent on Aβ structure. Soluble Aβ increased

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cholesterol content of Golgi whereas oligomeric Aβ reduced cholesterol content. However, the direction of effects of soluble Aβ on Golgi cholesterol content was contingent on the region of the Golgi complex. Aβ species have been shown to be generated in the Golgi complex (49) and a consequence of Aβ generation could be modification of cholesterol homeostasis in the Golgi complex. Alterations in cholesterol could impact on important Golgi functions such as protein sorting, sphingomyelin synthesis and lipid trafficking resulting in cellular pathophysiology. There is a reciprocal synergy between Aβ and cholesterol. Changes in cholesterol content alter Aβ expression and Aβ affects cholesterol homeostasis. The dynamic interaction of Aβ and cholesterol may be an important factor that contributes to cellular dysfunction occurring with AD.

Footnotes

2. The abbreviations used are: AD, Alzheimer's disease; $A\beta$, amyloid beta-peptide; APP, amyloid precursor protein; PC-PLD; phosphatidylcholine-phospholipase C; NBD-cholesterol, (22-(N-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3 $-\beta$-ol); HDL_3, high density lipoprotein$_3$; PC-PLC, phosphatidylcholine-phospholipase C; PKC, protein kinase C;

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Figure Legends

Figure 1. Localization of Cholesterol in Golgi of Astrocytes Incubated with Soluble and

Oligomeric $A\beta_{1-42}$. Confocal images of control astrocytes labeled with NBD-cholesterol (*panel*

a; green) and BODIPY TR ceramide (*panel b; red*). The overlay is shown in *panel c*. Confocal

images of astrocytes treated with soluble $A\beta_{1-42}$, NBD-cholesterol (*panel d; green*) and BODIPY

TR ceramide (*panel e; red*). The overlay is shown in *panel f*. Confocal images of astrocytes

treated with Oligomeric $A\beta_{1-42}$, NBD-cholesterol (*panel g; green*) and BODIPY TR ceramide

(*panel h; red*). The overlay is shown in *panel i*.

Figure 2. Percent Overlay of BODIPY TR Ceramide and NBD-Cholesterol in Astrocytes

Incubated with Soluble and Oligomeric $A\beta_{1-42}$. Quantitative measurements of the overlay of

NBD-cholesterol and BODIPY TR ceramide were determined using MetaMorph software and

expressed as percent overlay. Data are means ± SEM (n = 3 separate experiments). $^*p \leq 0.01$,

$^{**}p \leq 0.001$ as compared with control astrocytes.

Figure 3. UDP-Galactosyltransferase Activity in Astrocyte Fractions. Activity of UDP-

galactosyltransferase was determined in the homogenate, G1 and G2 fractions of astrocytes as

described in the Experimental Procedures section. Values are the means ± SEM (n = 3).

$^*p \leq 0.01$.

Figure 4. Fluorescence Intensity of Bodipy TR Ceramide in Astrocyte Fractions. Bodipy TR

ceramide was incubated with astrocytes for 1 h after which time cells were harvested and Golgi

fractions isolated using sucrose density centrifugation as described in the Experimental

Procedures section. Fluorescence intensity of BODIPY TR ceramide was measured in a

microplate reader using an excitation wavelength of 568 nm and emission wavelength of 598

nm. Fluorescence intensity was corrected for background fluorescence. Values are the means ± SEM (n = 3). $^{*}p \leq 0.001$.
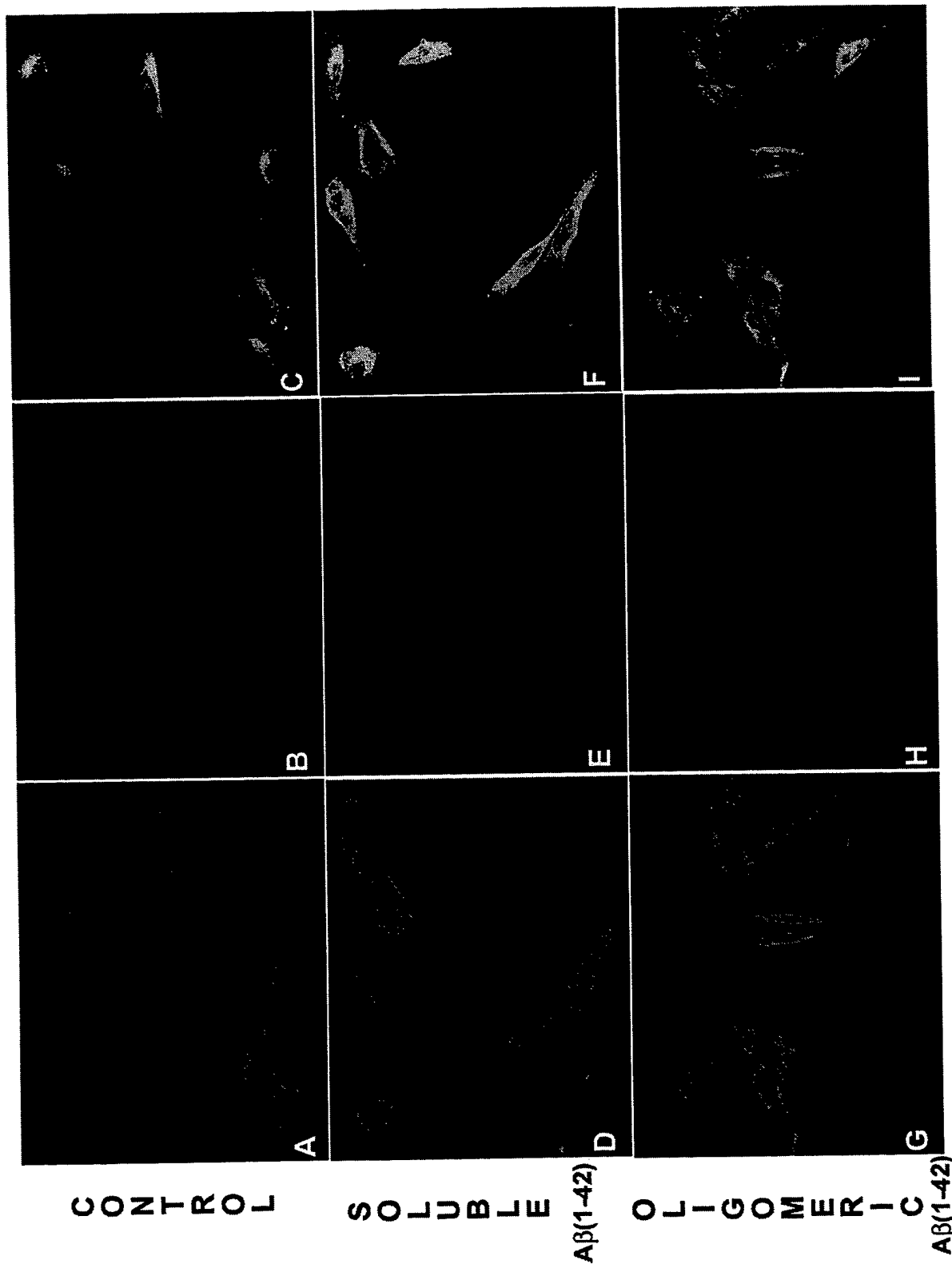
Figure 5. Distribution of Cholesterol in Astrocytes Incubated with Soluble and Oligomeric $A\beta_{1-42}$. Astrocytes were incubated with $A\beta_{1-42}$ for 2 h after which time cells were harvested and the Golgi complex isolated using sucrose density centrifugation as described in the Experimental Procedures section. The G1 fraction is the band at the 0.8-1.0 M sucrose gradient and G2 fraction is the band at the 1.0-1.2 M sucrose gradient. Cholesterol was determined enzymatically. Values are means ± SEM (n = 3). $^{+}p \leq 0.001$ as compared with the control G1 fraction; $^{*}p \leq 0.001$, $^{**}p \leq 0.0001$ as compared with control fractions.

Figure 6. Soluble and Oligomeric $A\beta_{1-42}$ Inhibit PC-PLD Activity in Astrocytes. Astrocytes were incubated with soluble $A\beta_{1-42}$ (panel A) and oligomeric $A\beta_{1-42}$ (panel B) for different time periods after which time cells were harvested as described in the Experimental Procedures section. Cells were placed in 96 well plates and PC-PLD determined using the Amplex® red PC-specific PLD assay kit (Molecular Probes, Eugene, OR). Fluorescence intensity was measured with a microplate reader using an excitation wavelength of 542 nm and emission wavelength of 590 nm. Values are means ± SEM (n = 3). $^{*}p < 0.001$ as compared with control cells; $^{+}p < 0.001$ as compared with 30 min incubation time.

NBD Cholesterol   BODIPY TR Ceramide   Colocalization

CONTROL

A   B   C

SOLUBLE Aβ(1-42)

D   E   F

OLIGOMER-C Aβ(1-42)

G   H   I

Figure 2

Percent overlay of BODIPY TR ceramide and NBD-cholesterol in astrocytes/mg cell protein
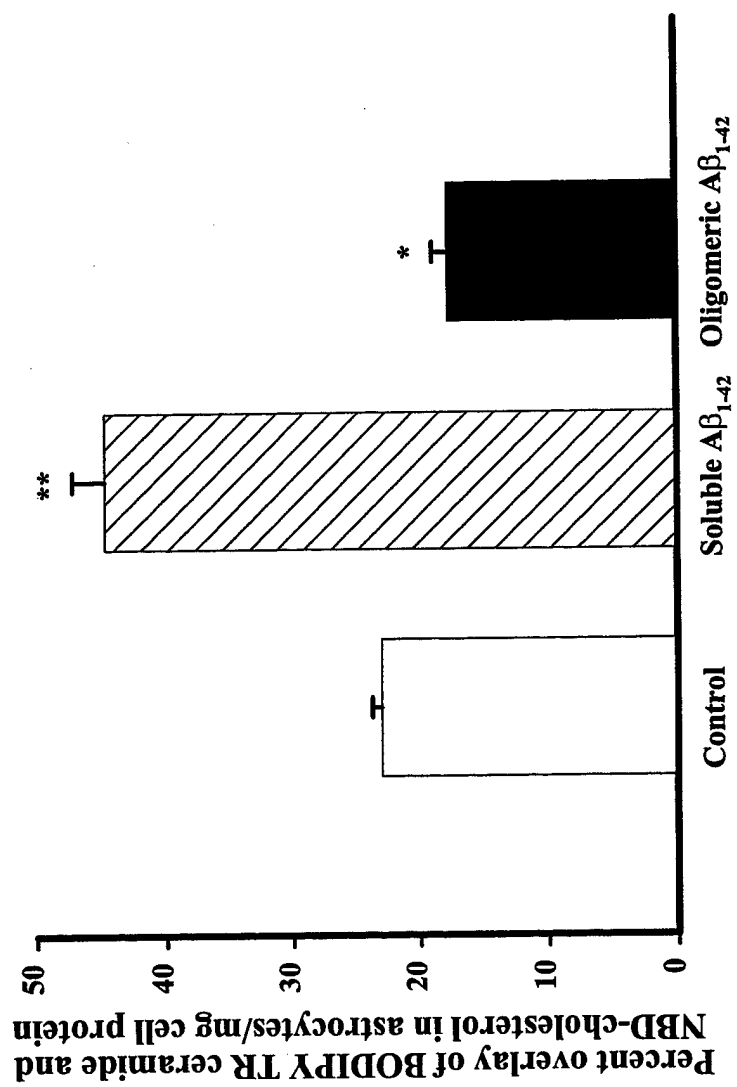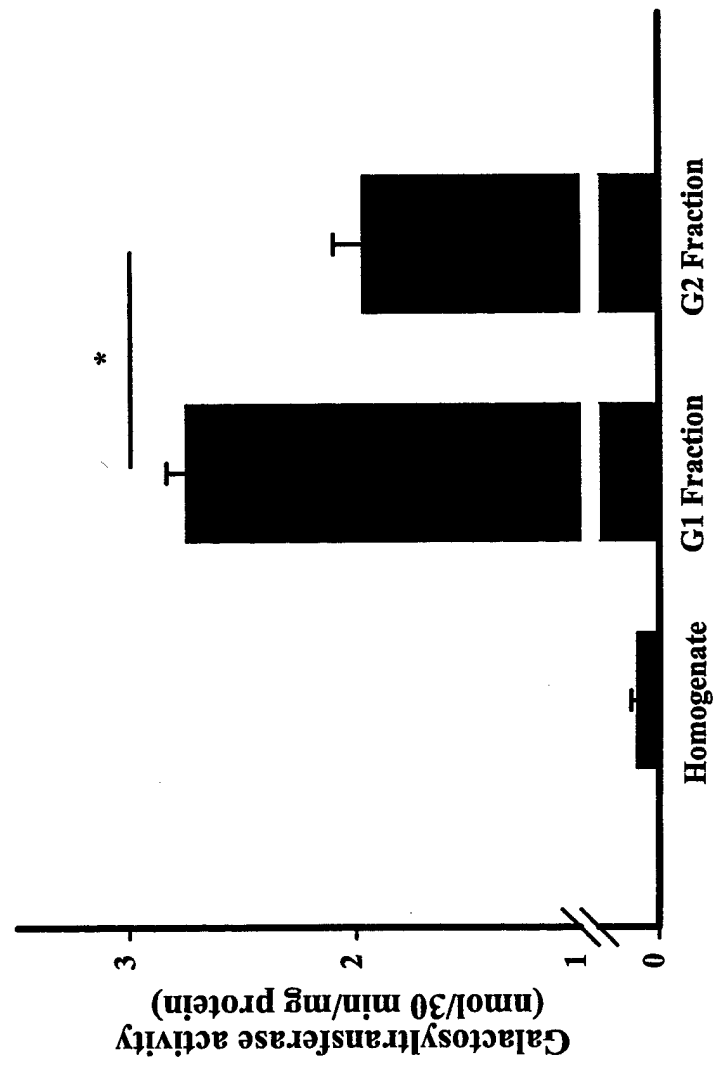
Control   Soluble Aβ₁₋₄₂   Oligomeric Aβ₁₋₄₂
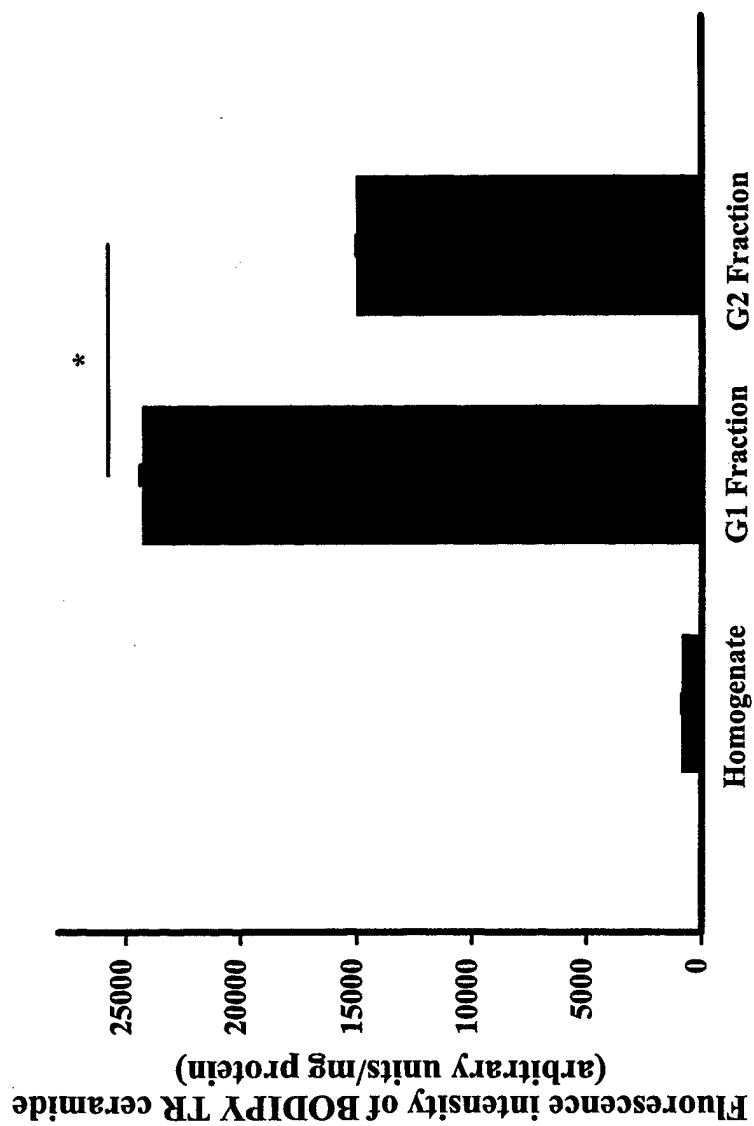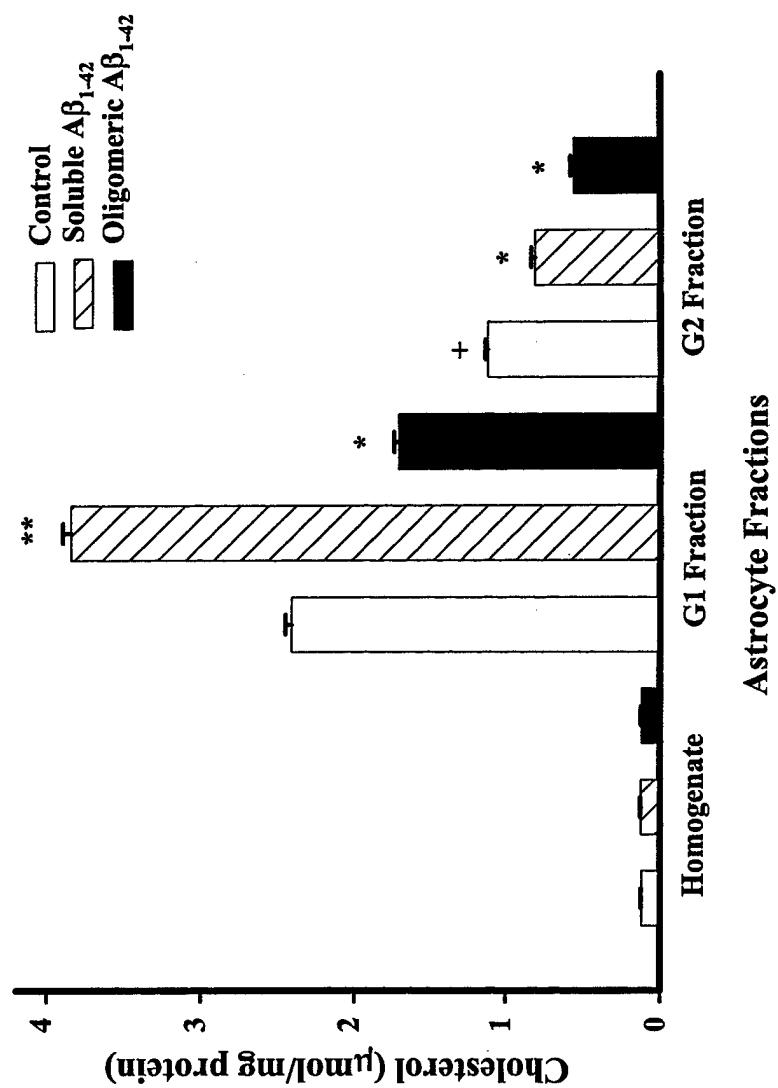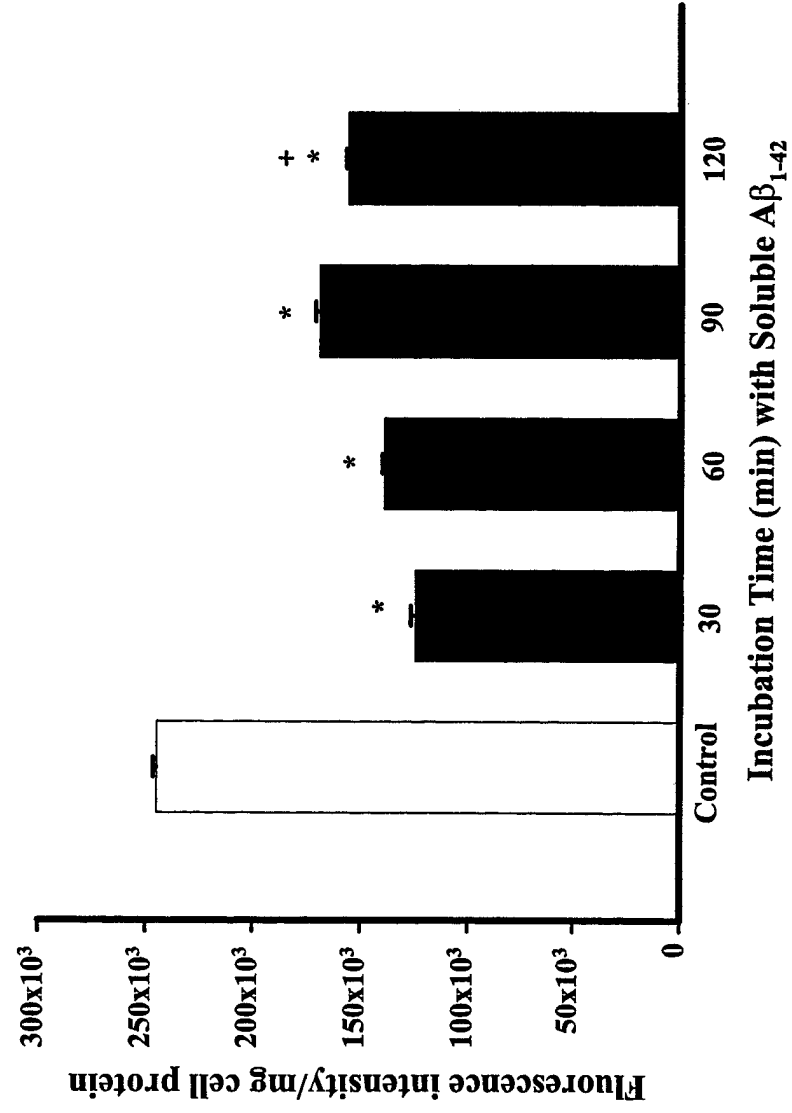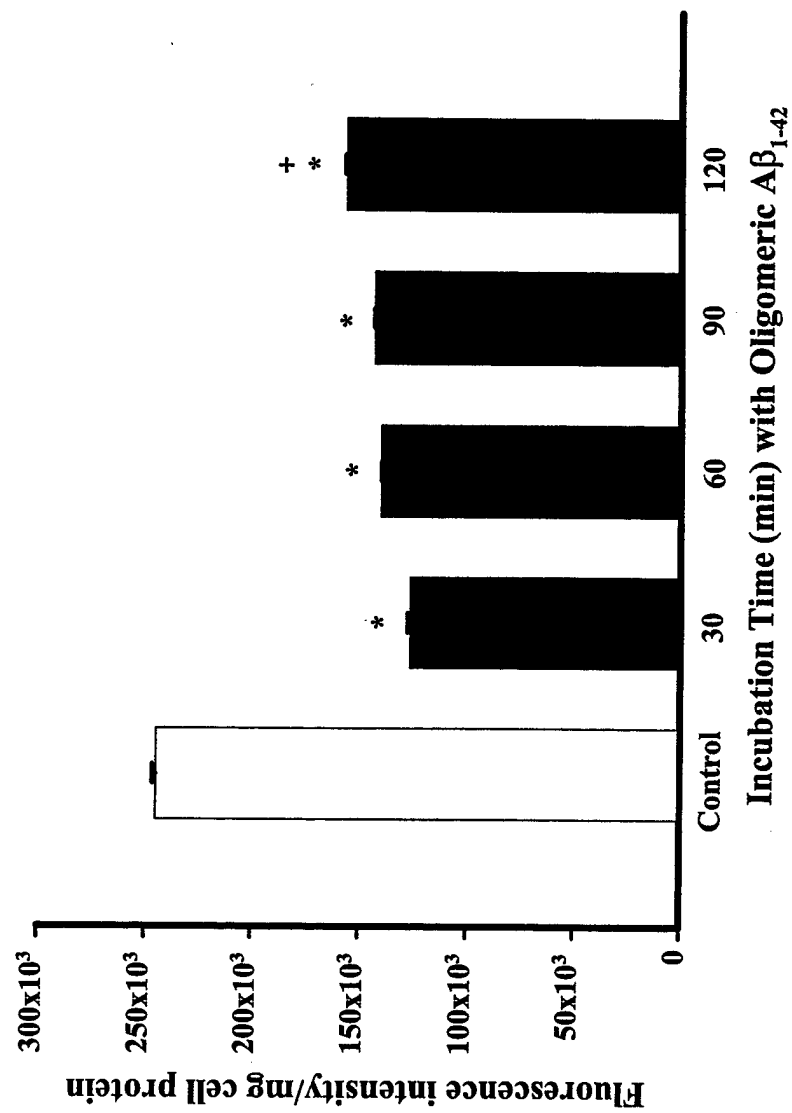
Figure 3

Figure 4

Figure 5

Figure 6A

Figure 6B

HDL AND APOLIPOPROTEIN AI-INDUCED CHOLESTEROL EFFLUX IS INHIBITED BY ETHANOL IN HUMAN SMOOTH MUSCLE AORTIC CELLS: ROLES OF PHOSPHOLIPASES C AND D

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Molecular mechanisms of ethanol-induced inhibition of cholesterol efflux are not well-understood. Cholesterol is removed from cells by HDL and lipid-free and lipid-poor apolipoproteins. The ATP binding cassette transporter is thought to play a role in cholesterol efflux by direct interaction with apoA-I but not HDL. There is also evidence that the Golgi complex contributes to regulation of cholesterol efflux that may involve activity of PC-PLD and PC-PLC. Effects of ethanol on cholesterol efflux may differ depending on whether efflux is elicited by HDL or apoA-I and activity of PC-PLD or PC-PLC. These hypotheses were examined using human smooth muscle aortic cells. Ethanol (50 and 75 mM) significantly inhibited HDL-mediated cholesterol efflux from cells. ApoA-I was more effective in inducing sterol efflux than was HDL with significant inhibition occurring at 25 mM ethanol. Both PC-PLD activity and PC-PLC activity were significantly affected by ethanol and ethanol directly altered cholesterol in the Golgi complex as revealed by confocal microscopy. Ethanol-induced perturbation of the Golgi complex in addition to altering cholesterol efflux may affect other important Golgi functions such as formation and release of secretory vesicles including lipid rafts and is a new approach to understanding cellular actions of ethanol. Supported by a grant from the US Army Medical Research and Material Command (DAMD 32001) and the Department of Veterans Affairs.